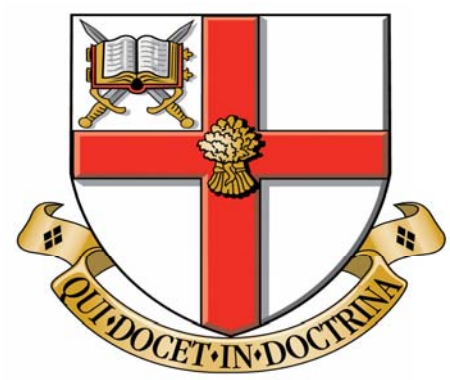

University of Chester

Genetic analysis of the critically endangered Trinidad Piping guan (*Pipile pipile*): Implications for phylogenetic placement and conservation strategies

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy

By: **Louise Anne Robinson**

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Abbreviations Used in the Text

AIC	Akaike Information Criterion
AFLP	Amplified Fragment Length Polymorphisms
AMNH	American Museum of Natural History
bp	Base Pairs
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
CITES	Convention on International Trade of Endangered Species of Wild Fauna and Flora
COI	Cytochrome *c* oxidase subunit I
Cyt *b*	Cytochrome *b*
ddH_2O	Double distilled water
dATP	Deoxyadenosine triphosphate
ddNTP	Dideoxynucleotide Tri-phosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
Defra	Department for Environment, Food and Rural Affairs
D-loop	Displacement loop
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FINS	Forensically Informative Nucleotide Sequencing
g	Gram
HCl	Hydrogen Chloride
HV	Hyper-Variable
ISIS	International Species Identification System
ISFG	International Society for Forensic Genetics
IOC	International Ornithological Congress
IUCN	International Union for Conservation of Nature

Kb	Kilobases
KCl	Potassium Chloride
Mins	Minutes
ml	Millilitre
mtDNA	Mitochondrial DNA
MgCl	Magnesium Chloride
MYA	Million Years Ago
NCBI	National Centre for Biotechnology Information
nDNA	Nuclear DNA
ng	Nanogram
nm	Nanometer
NOAA	National Oceanic and Atmospheric Association
PCR	Polymerase Chain Reaction
pmol	Picomole
PSG	Pawi Study Group
RNA	Ribonucleic acid
RFLP	Restriction Fragment Length Polymorphisms
SACC	South American Classification Committee
SMM	Sabah Museum
SNP	Single Nucleotide Polymorphism
tRNA	Transfer RNA
TAE	Tris-acetate-EDTA
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
TWGDAM	Technical Working Group on DNA Analysis Methods
UV	Ultraviolet
UWI	University of West Indies
V	Volt
WPA	World Pheasant Association
µl	Microlitre

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Abstract

Genetic analysis of the critically endangered Trinidad Piping guan (*Pipile pipile*): Implications for phylogenetic placement and conservation strategies

Robinson, L. A.

Classified as critically endangered since 1994, the Trinidad Piping guan (*Pipile pipile*) is an endemic species estimated to number less than 200 individuals. Known to locals of Trinidad as the 'Pawi' this bird has been the subject of substantial hunting pressures and much of the species habitat has been destroyed through deforestation. Although officially protected since 1958, occasional recreational hunting of this elusive species still occurs. Due to difficulties locating and capturing the species, no genetic research has previously been performed using samples obtained from Trinidad. All previous research studies have been conducted using biological materials obtained from captive birds outside Trinidad and island data has never been obtained or compared. The genetic diversity of the remaining population was therefore examined through the investigation of mitochondrial haplotypes, pairwise comparison and SNP analysis. With the intention of assisting the protection of this endangered species by the location of remaining areas of habitation, methods of genetic identification were established for the Trinidad Piping guan utilising non-invasive feather samples.

Species specific primers were created in the regions of the ND2 and cyt *b* genes of the mitochondrial genome to identify *Pipile pipile*. Species detection was further verified with the use of PCR-RFLP of the same gene regions digested with *BsaXI*, *EcoRV* and *BsrDI*. This combined approach allowed the separation of closely related taxa based on single inter-species SNPs. Confirmation of species identification was subsequently performed through the use of forensically informative nucleotide sequencing. The established methodologies were used in the current study to correct the classification of a UK breeding population of Piping guans thought to be *Pipile pipile* and to identify Trinidad field samples. These detection methods have implications for ecological studies through the location of populations from trace evidence collected in the field. In addition this method could be used to assist Trinidadian police forces in the identification of bushmeats or simply act as a deterrent to hunters.

The sequence data obtained in the present study were also used to re-assess the phylogeny of Piping guans. As genetic sequence from a true island bird was previously unstudied, differences between phylogenies created using non-island and island bird data sets were examined. Combined analysis was performed on 1884bp of the ND2 and cyt *b* genes and placement of Trinidad Piping guan was found to differ from that which has been previously published.

Chapter 1

Conservation and Status of the Trinidad Piping guan

1.1 Cracids

The family of Cracidae lies within the Order of Galliformes and contains three groups, Chachalacas, Guans and Curassows. Each group comprises a number of genera, the group Chachalaca contains only *Ortalis* whereas Guans consist of *Penelope*, *Chamaepetes, Oreophasis, Penelopina, Aburria* and *Pipile* with Curassows containing *Pauxi, Crax, Nothocrax* and *Mitu* (Delacour, Amadon, del Hoyo & Motis, 2004). In total the family contains 50 species (of which 13 are threatened).

The distribution of Cracids is found in the Neotropics, from the southern regions of Texas, United States through to the Paraná Delta of Uruguay and Central Argentina. Sizes within the family range from a length of 42cm (Buff-brown Chachalaca) to 92cm (Great Curassow) with a similar appearance to other gallinaceous birds (del Hoyo, Elliot & Sartagal, 1994). All species possess a long neck and small head which often contains a crest of feathers varying in size and curvature between species. In addition, they have long, strong legs, a long tail, rounded wings and an elongated hind toe which is used to grip branches when perching in trees (del Hoyo et al., 1994). Flying is limited to short bursts and to gain height before gliding or to escape predation. Of all genera, the Piping guans (*Pipile*) are the more superior fliers as they are a slender build and therefore have a lesser body weight. Flight is not sustained but the genus can achieve greater distances than that of larger birds such as the Curassows, which can be twice the weight (del Hoyo et al., 1994).

Cracids play an important role in maintaining preferred foods within tropical forests through the process of seed dispersal, therefore replenishing plants within suitable habitat (Brooks & Strahl, 2000). All Cracids are hunted but it has been shown that they are unsuitable for sustained hunting due to a slow reproductive rate (Begazo & Bodmer, 1998) leading to population decline.

1.1.1 The Piping guans

The Piping guans average 60cm in length and all have a similar appearance, the most distinctive morphological feature is that of the dewlap which differs in colour between species. Over nearly 80 years it has been suggested that the genus contains varying numbers of species and their relation to each other debated. Previous publications have seen the inclusion of subspecies, conspecific species, sympatric species, super-species as well as hybridisation where habitats overlap (McCarthy, 2006) and the merge of *Aburria* with *Pipile* (Delacour & Amadon 1973; Pereira, 2000a; Grau, Pereira, Silveira, Höfling & Wajntal, 2005; Frank-Hoeflich et al., 2007).

However, the four species currently recognised by the International Union for Conservation of Nature (IUCN) Red List, the International Ornithological Congress (IOC) and Bird Life International are *Pipile pipile* (Trinidad Piping guan), *Pipile cumanensis* (Blue-throated Piping guan) including *P. c. cumanensis* and *P. c. grayi*, *Pipile cujubi* (Red-throated Piping guan) including *P. c. cujubi* and *P. c. nattereri* and finally *Pipile jacutinga* (Black-fronted Piping guan) (del Hoyo et al., 1994; Brooks & Strahl, 2000). Today, this classification is not accepted by all, with the genus of *Pipile* sometimes treated as a single species of common Piping guan - *Pipile pipile* (Hilty, 2003). The geographical distribution of Piping guans can be seen in figure 1.1 taken from Delacour et al., (2004); this information is based on one of the disputed classifications of two species of Piping guan using the *Aburria* synonym.

Fig 1.1: Map showing the ranges of all species of Piping guan taken from Delacour et al., (2004). Ranges of the superspecies Aburria [Pipile]. *1. Common Piping guan* (Aburria pipile). *The subspecies are: 1a.* pipile; *1b.* cumanensis; *1c.* grayi; *1d.* nattereri; *1e.* cujubi. *2. Black-fronted Piping Guan* (Aburria jacutinga).

1.1.2 Disputed classification of the Piping guans

Classification of the Piping guans has caused significant debate, having in the past been included, for example, within *Penelope* and also for a separate tribe of *Pipilinae* to be created to include relatives of *Aburria* and *Chamaepetes*. In 1973, Delacour & Amadon made a recommendation based on morphological characteristics that *Pipile* should merge with the earlier synonym *Aburria*. Though it has since been suggested that this merge was incorrect and the two are in fact sister genera (Pereira, Baker & Wajntal, 2002) more recent genetic

data support the merge of *Pipile* into *Aburria* (Pereira, 2000a; Grau et al., 2005; Frank-Hoeflich et al., 2007).

Although Piping guan classification is now frequently published as *Aburria*, this merge has not been supported by all and was rejected by the South American Classification Committee (SACC) in 2007 (Remsen et al.). The rejection of this proposal was based on two main factors, the first being the low Bayesian nodal support values for *Aburria* and the second that only one individual of each species was used for representation, none of which were voucher specimens. In this respect, the study was not considered to be scientific (i.e. not repeatable) as without voucher specimens there is the possibility that a hybrid or back-cross individual was sampled. However, the collection of voucher specimens is not always possible, especially when studying threatened species (discussed further in Chapter 4). Researchers frequently rely on donated samples, as did the study on which this 2007 proposal was based. In the current study, the comparison of *Pipile pipile* to other species of Piping guan also relies to some extent on previously collected data from non-voucher specimens.

1.1.3 Current phylogeny of the Piping guans

Phylogenetic assessment of Neotropical Piping guan dispersal was performed by Grau et al., (2005) and includes a single representative of the species *Pipile jacutinga*, *Pipile cujubi*, *Pipile pipile*, *Pipile cumanensis*, the wattled guan *Aburria aburri* and out-group species *Penelope obscura* and *Penelope superciliaris* (see figure 1.2). No sub-species were included in this study but it is assumed that placement would be adjacent to the species from which it branches. Prior to this, Piping guans had only been included in phylogenetic studies to the family level of Cracid species (Pereira et al., 2002; Pereira & Baker, 2004) and therefore the placement of individual species of Piping guan within a phylogeny was unknown.

Fig. 1.2: Modified phylogeny from Grau et al., (2005). Bootstrap values using maximum parsimonious and maximum likelihood methods for each node are indicated on the left of the tree respectively.

The tree constructed by Grau et al., (2005) places *Pipile pipile* between *Pipile cujubi* and *Pipile cumanensis*. The divergence time calculated in this publication (0.4 - 1.6MYA) is inconsistent with the geographical age of Trinidad but dispersal from mainland was considered to be a plausible hypothesis. This would have occurred during regression of glacial sea levels revealing the continental platform between Venezuela and Trinidad which lies 200m below sea level. The samples obtained from *Pipile pipile* which were used to create this phylogeny were obtained from a captive bird at the aviary Granja 'La Siberia' in Mexico and as such no previous genetic investigation has ever been conducted on the species using samples obtained from a true island bird. Moreover, this sample is neither a voucher representative nor reliable reference as no data is publically available regarding this bird. Despite trying to contact the owner of the aviary in Mexico no reply to queries was ever received and samples could not be obtained, this being the issue of repeatability mentioned

by Remsen et al., (2007). The placement of *Pipile pipile* has therefore been disputed but due to a lack of alternate data, the position of the species within the phylogeny has not previously been reassessed. Data obtained in the current study from an island bird therefore gave the opportunity to create a phylogeny and examine the inconsistent divergence time previously observed.

1.1.4 Geographical distribution of the Trinidad Piping guan

The Trinidad Piping guan known locally as the 'Pawi' is an endemic species found only on the Caribbean island of Trinidad off the mainland of South America (location 1a in figure 1.1). A monotypic island species, the Trinidad Piping guan has suffered a severe decline in population numbers in the last century due to the effects of habitat loss and hunting. Because of the species low reproductive rate and clutch size, hunting has led to unsustainable population numbers and near-extinction (Brooks, 1998). It is suspected that less than 200 individuals remain (Bird Life International, 2010), classifying the species as Critically Endangered. This defined the species population as seeing a decline of 80% within three generations and therefore threatened with extinction (IUCN, 2010). The species is listed in Appendix I of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) (IUCN, 2010) which dates back to 1994 when this threat category was applied to avian species (Nelson et al., 2011). The last estimate of population size was conducted in 1998 and although attempts were made to re-estimate population size in 2005 using distance sampling, the method was found to be unsuitable. No birds were located during the pilot study and as such no full survey was subsequently conducted (Keane et al., 2005). Research carried out in the 1980s suggested that the species was well distributed throughout Trinidad until the 1940s. It has been speculated that after this time an increase in the occurrence of hunting and deforestation led to the significant population decline (James & Hislop, 1988) demonstrated in figure 1.3 taken from Hayes, Sanasie & Samad (2009a). The entire population of the Trinidad Piping guan is now thought to be located in and around an area of 250km^2 of protected habitat (of which 150km^2

is within Matura National Park) in the Northern Range of Trinidad. At the time of study by James & Hislop (1988) it was believed that two separate populations existed, that of the remaining population found within the Northern Range and a small population in the Southern Range. The last sighting in the Central Range was in 1983 and it is suspected that the species no longer resides in this area due to increased human encroachment (Hayes et al., 2009a). Sightings within the southern regions were recorded in 2000; however no subsequent search has been conducted within the Southern and Central ranges in order to confirm these observations (Hayes et al., 2009a).

Fig. 1.3: Map of Trinidad displaying the historical distribution of Pipile pipile *based on the last reported sighting within each location (Hayes et al., 2009a).*

1.1.5 Morphology of the Trinidad Piping guan

The Trinidad Piping guan is black/brown with purple/brown gloss colouring, long white crest feathers and white colouration surrounding the eyes. The upper wing coverts are either white or white-tipped and the sides of the face, throat and dewlap are cobalt blue and legs red (Delacour et al., 2004). An illustration of a Trinidad Piping guan can be seen in figure 1.4.

Fig. 1.4: A free-living Trinidad Piping guan displaying the distinctive morphological characteristics of the species.

Photograph provided by Kerrie T. Naranjit.

1.1.6 History and ecology of the Trinidad Piping guan

The Trinidad Piping guan is described as a canopy bird, demonstrated by an ecological study conducted by Hayes et al., (2009b) in which six Trinidad Piping guans were observed intermittently between May 1997 and July 2001. When observed they were seen to spend 93.6% of time in the forest canopy >5m above ground, 6.2% of time between 0.1-5m above ground and only 0.2% of time on the ground (also supported by a two year field study conducted by Naranjit, 2010a). Early records of the species mention flocks walking on the forest floor but this is now rarely seen.

Its vocalisation is a 'piping' of whistles ascending in pitch, mainly heard during the breeding season with a variety of other 'conversational' calls (Delacour et al., 2004). The diet of the Piping guan mainly consists of fruits and seeds which through the process of dispersal helps to maintain forest vegetation (Sedaghatkish, Galetti & Denny, 1998).

There are many early records which describe the consumption rather than conservation of the species, for example De Verteuil (1858) wrote that the species when compared to a pheasant had *'the advantage of being far more juicy'* (pp436) and *'…is very stupid; so much that, if several are met with together, they can be shot in succession, as they do not fly, even after their companions have been killed'.* (pp122). In 1894, Chapman wrote that *"the flesh of this species is deservedly esteemed, and through the persecution of hunters it is rapidly becoming a rare bird"*. One hundred years later the species was classed by the IUCN as Critically Endangered. The use of guns in hunting may go some way to explain why, despite centuries of hunting pressures, the species was still found on the forest floor some 200 years ago but is now classed as a canopy bird. If early records are assumed correct, a change in behaviour could be interpreted (although evidence for this is limited due to the source of early observations) from forest floor to arboreal behaviour. However, the species is generally unperturbed by human presence and will quickly become habituated if canopy trees remain and the bird is not hunted (Hayes et al., 2009b).

1.2 Conservation of the species

Although the Trinidad Piping guan has been officially protected since 1958 as part of the Conservation of Wildlife Act (Hayes, 2006) hunting remained frequent for the next forty years (Temple, 1998). However, because of recent conservation efforts, it is thought that hunting has declined and from research conducted by Hayes et al., (2009a) locals claim to no longer actively hunt the Trinidad Piping guan. It is clear however that hunting does still occur (perhaps taken opportunistically when hunting other species) as in December 2010 a live Trinidad Piping guan was seized, having four pellet shot wounds to the wing and an injury to one eye. The bird was treated by veterinarians in Trinidad but is not currently fit for release (John Cooper, personal communication, January 17, 2011). This incident suggests that hunting may still pose a threat to the species survival, especially if birth rates of the species are low and mortality is greater or equal to the rate of reproduction.

Currently, there appears to be no pure bred member of the species present in captivity. The two last recorded captive specimens in Trinidad were held at the Pointe-à-Pierre Wild Fowl Trust and Port of Spain Zoological Garden. The former was killed by dogs (Connolly & Seutin, 1998) and the latter specimen died of natural causes during the course of the current study. Should the aforementioned shot bird be unable to be released, it will remain the only captive specimen.

1.2.1 Raising public awareness

Since the creation of the working party, the Pawi Study Group (PSG) in 2004 based at the University of the West Indies (UWI) in Trinidad, supported by the World Pheasant Association (WPA) in the UK (Cooper & Cooper, 2007; Pawi Study Group, n.d.), the Trinidad Piping guan has gained the interest of researchers and has been the focus of awareness campaigns. This working group of zoologists and veterinarians (The University of West Indies, n.d.) was developed as part of a project initiated by Prof. John Cooper to co-ordinate health studies as well as research of the behaviour, habitat and local opinion of

the Trinidad Piping guan (Cracid Specialist Group, 2004). In the six years that the project ran, public awareness of the species and its threatened status was raised through the introduction of educational campaigns (see figure 1.5). Information boards were also placed around Trinidad and health assessments were conducted on the captive Trinidad Piping guan which was held in the Port of Spain Zoological Garden.

Fig. 1.5: Example of a leaflet titled 'The Story of Pawi' forming part of an educational campaign. Leaflet provided by Prof. John Cooper.

1.2.2 The effect of awareness campaigns on attitudes

In 2009, a survey conducted by Waylen, McGowan, Pawi Study Group & Milner-Gulland in the village of Grande Riviere (located within Trinidad Piping guan habitat) found that although hunting was perceived by community members to be a threat to wildlife, a third of all households contained one or more hunters. Hunting was considered a pastime or of cultural importance by 69% of these households, not a necessity. Though wild meats were preferred, these were eaten less than once a month and meat from the Trinidad Piping guan was not prized but perhaps taken opportunistically whilst hunting other species. Therefore, although those living in the Grande Riviere region recognised that hunting was the biggest threat to wildlife and 77% of persons questioned claimed there *'should be a little more effort'* or *'should be much more effort'* put to conserving Trinidad's plants and animals, these attitudes did not necessarily impact on pre-existing hunting behaviours. Awareness of Trinidad Piping guan conservation efforts were generally appreciated to a lesser extent than that of the critically endangered leatherback turtle (*Dermochelys coriacea*). No correspondents mentioned the species when questioned about conservation issues which required more attention and only 14 of the 52 participants named the Trinidad Piping guan as a threatened species (Waylen et al., 2009). The effect of ecotourism and conservation activities appeared to be related with participants' knowledge and therefore increasing these initiatives for the Trinidad Piping guan may aid the species survival through awareness.

It is hoped that an increase in ecotourism will generate income and therefore prompt a behavioural change towards the species. A poster campaign to raise awareness of the plight of the Trinidad Piping guan can be seen in figure 1.6 to contain bullet holes. This could be interpreted as an attitude to the species conservation but could equally have been unintentional or the sign used for target practice.

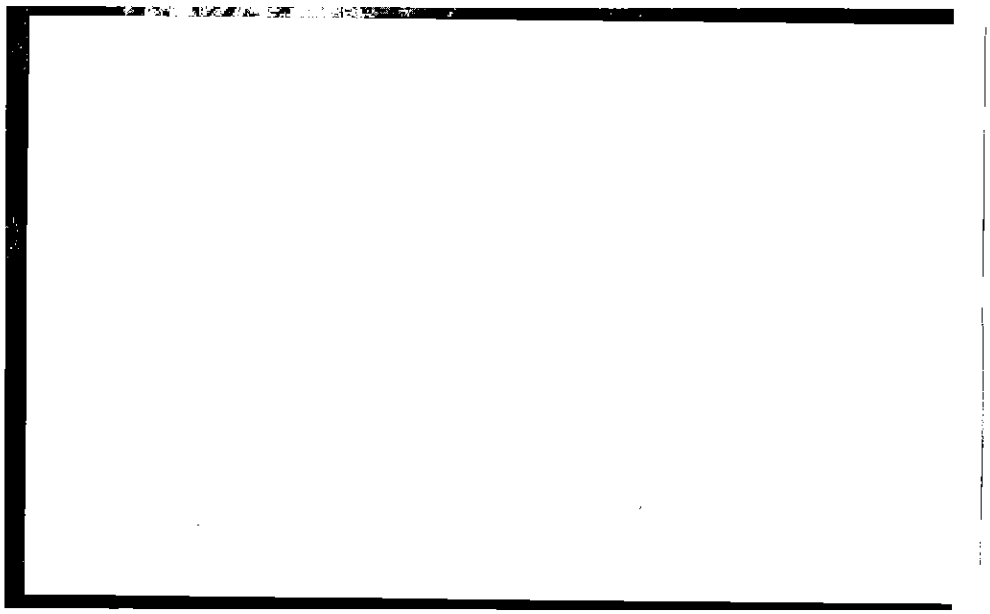


Fig. 1.6: A poster campaign raising awareness of the plight of the Trinidad Piping guan in which bullet holes can be seen.
Photograph provided by Kerrie T. Naranjit.

1.2.3 Ecotourism

Setting up tours and employing guides in order to protect species can be difficult to implement and is generally only effective if previous hunting of the species was for commercial gain rather than sport or food. Being Trinidad's only endemic bird, the Trinidad Piping guan does attract the interest of some bird watchers (see figure 1.7) whose travel to the island helps generate local income via the leisure and tourism industry (Waylen, et al., 2009).

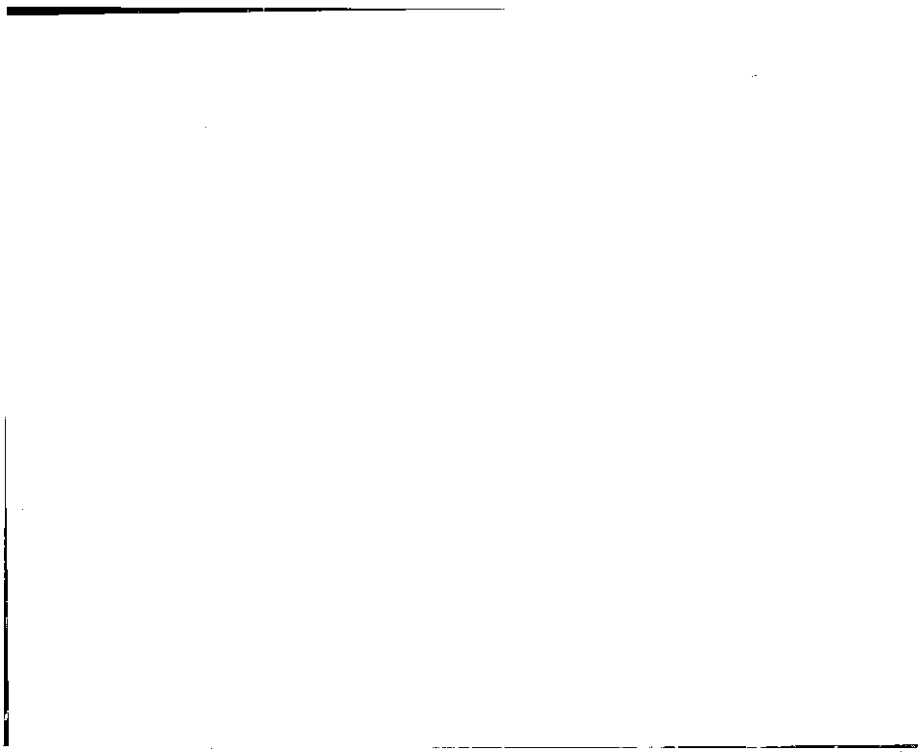


Fig. 1.7: Eco-tourists observing Trinidad Piping guan in its natural habitat.
Photograph provided by Kerrie T. Naranjit

Similar projects have been set up worldwide but the importance of establishing the mechanisms of ecotourism specifically tailored to the species of concern is imperative in order for local communities to profit (Bookbinder, Dinerstein, Rijal, Cauley & Rajouria, 1998; Tisdell & Wilson, 2001). In this respect, local residents with knowledge of the forests can be encouraged to act as tour guides (Brooks & Strahl, 2000) which would hopefully reduce hunting if populations are shown to have the potential to generate income. However, a number of large organisations currently field the market in birding expeditions which include possible sightings of the Trinidad Piping guan. These trips can include either day excursions to Trinidad or if overnight stays are involved, accommodation is provided at locations such as the Asa Wright Nature Centre. A new (2012) Guyana and Trinidad excursion is being established by International Expeditions (http://www.ietravel.com/central-south-america/guyana-trinidad) which includes

a three night stay at this Centre. In addition, if travelling at the correct time of year, excursions are available to see nesting leatherback turtles.

This income although positive for this not-for-profit trust, removes the generation of income for local residents through hotel stays and possibly guided tours. As these companies already employ specialist guides, it would not be beneficial for them to employ local residents to conduct the same expeditions. Due to the elusiveness of the Trinidad Piping guan, sightings can be unpredictable and therefore an expedition based solely on this species may be unlikely to attract tourists. These issues surrounding the ecotourism of the Trinidad Piping guan are currently being considered as there is positive interest from locals to get involved and generate income. Solutions to these issues need to be found which are cost effective, benefit as many members of the community as possible, attract substantial tourist interest and do not directly compete with places such as the Asa Wright Nature Centre. One possibility would be for the Pawi Study Group to work in collaboration with these tours to provide specialist knowledge regarding the endangered status of the species and current conservation research. If guided tours have been unsuccessful in sightings, the bird which is currently in captivity would provide an opportunity for a direct and close sighting. This captive viewing may not be optimal for keen bird watchers but could provide an opportunity for close observation.

If regular tours were able to be established, the frequent visiting of known areas provides opportunity for monitoring populations with regard to size and birth rate. The 2009 survey conducted by Waylen et al., found that 25% of the community were indirectly involved in ecotourism surrounding the leatherback turtle and although not involving every household, those participants were likely aware of the potential benefits of ecotourism for themselves, friends or family. If this can be translated to the Trinidad Piping guan, it may help reduce hunting pressures and aid conservation.

1.2.4 Field based research

UWI undergraduates and postgraduates as well as UK postgraduates from Imperial College have conducted ecological and behavioural investigations on the species as MSc research (Cooper & Cooper, 2007). A two year field study (2008-2010) has enabled information to be gathered on factors such as group dynamics, nesting habits and diet to assist the development of species recovery plans (Naranjit, 2010b). One unsuccessful avenue of investigation which has been attempted is the capture of individuals using mist nets. It was proposed that this would allow health assessments to be conducted and radio collars fitted to determine range (see figure 1.8). Unfortunately, despite using a decoy and birds flying within metres of nets, none have been captured to date (Hayes et al., 2009b; Naranjit, 2010b).

Fig. 1.8: Mist net and decoy erected in Trinidad Piping guan habitat.
Photograph provided by Kerrie T. Naranjit

1.2.5 The use of species field markers in Trinidad

Due to the highly elusive nature of the species, demonstrated not only in the lack of sightings but also the inability to capture individuals in mist nets, one aspect of genetic study which would be of great assistance to researchers in Trinidad is the creation of field markers to identify the species. Currently identification relies on sightings, which as demonstrated by the pilot study conducted by Keane et al., (2005) are rare. If methods are available which allow the detection of the species from trace evidence, identification would be possible without direct sightings. This may therefore be of use to researchers identifying whether the species remains within the central and Southern regions of the island, thereby also detecting habitat which requires protection. This method of species identification may have many implications for the recently published Pawi Species Recovery Strategy (Nelson et al., 2011) with respect to species distribution, the range of habitat in which it resides and tolerance to habitat alteration by the identification of birds in these areas. The collection of trace evidence is also a cost-effective approach because although the regularity of samples is unknown, they can be collected by researchers or locals opportunistically as no training or specialist equipment is required. This may therefore increase the volume of samples obtained and the likelihood of identifying a Trinidad Piping guan feather. If collection of materials is encouraged throughout Trinidad, this provides opportiunity for continual spatial monitoring of the species. Mist netting has so far been ineffective and therefore a non-invasive approach may accumulate samples, and consequently data, faster than invasive methods. However, aspects which cannot be considered by this approach are health and population studies of the species of which alternate methods will be required.

Morphological identification of trace evidence such as feathers can be difficult to conduct as distinguishing between species with similar plumage may require knowledge of issues such as barbule analysis. However, genetic identification provides a reliable method of species detection and can quickly eliminate non-target species. Species identification has been achieved using mitochondrial

DNA (mtDNA), the properties of which readily lend themselves to the extraction of material from samples recovered in the field. The identification of such markers was therefore achievable during the course of the current investigation as multiple samples became available.

1.2.6 Combating wildlife crime

In Trinidad, the Forestry Division and local police forces are relied upon to enforce conservation regulations. In the previously mentioned case of the seized Trinidad Piping guan, the bird was rescued by Trini Eco Warriors who contacted Blanchisseuse police for assistance (see figure 1.9). Regulations are difficult to enforce and prosecuation is problematic if carcass remains cannot be identified morphologically. Methods of genetic identification could therefore be used in forensic circumstances to identify meats derived from the species..

Fig. 1.9: Trinidad Piping guan being transported to Blanchisseuse police station which was later transferred to the North West Veterinary Clinic, Port of Spain.
Photograph provided by Marc de Verteuil

1.3 Forensic Techniques

As with human forensics, wildlife forensics has advanced in parallel to the formation of new genetic methodologies through pioneering techniques and the progression of laboratory technology. In wildlife investigations, the four main areas for genetic study are species identification, geographic locality, individual identification and DNA sexing (Alacs, Georges, FitzSimmons & Robertson, 2009). Whereas the latter two fields purely involve the study of the nuclear genome, mtDNA displays variation which is sufficient for species identification (except in hybrid species due to maternal inheritance) and can be used to identify geographic location if mitochondrial haplotype variation is sufficient.

Most methods used to investigate wildlife crime have been adapted from techniques of human forensic science and therefore closely follow the advances in this field. Two years after the first human genetic fingerprints were created; Burke and Bruford (1987) used the same technique to demonstrate familial similarities in birds. In 1989 Thommasen, Thomson, Shutler & Kirby established nuclear DNA (nDNA) fingerprint methods for wildlife identification. Although the DNA fingerprints of some species had been established, it was thought due to introns and recombination effects that the use of fingerprinting techniques was unsuitable for identifying species. In 1991, Cronin, Palmisciano, Vyse & Cameron utilised mtDNA for species identification by molecular taxonomy, believing it to be more suitable for this application due to its characteristics and mode of inheritance. Though some specific knowledge is required within the investigation of wildlife crime, the standard forensic protocols used within human investigations such as contamination, storage and continuity of evidence are still of the utmost importance, especially for the presentation of evidence in court (Cooper, Cooper & Bugden, 2009).

1.3.1 Mitochondrial DNA

The mitochondrial genome is an extranuclear circular molecule 15-20 kilobases (kb) in length, varying in size between species. Variation in the size of the genome normally occurs due to the length of the non-coding region called the displacement loop (D-loop), also known as the control region. In avian species the genome averages 16.5kb, the first of which was established by Desjardin & Morais in 1990 using chicken (*Gallus gallus*) as a model species. There are 37 genes within the mitochondrial genome (see figure 1.10), 13 of which code for subunits of the electron transport chain (protein coding genes), 22 transfer RNA (tRNA) and 2 ribosomal RNA (rRNA) genes (Ballard & Whitlock, 2004). All vertebrate mtDNA contain the same number of genes which can differ in their arrangement. The gene order of Aves varies from the standard mammalian order in the genes surrounding the control region, this variance is thought to have arisen during the bird-crocodilian split 254 million years ago (MYA), (Pereira, 2000b). In addition, there are two known gene orders which occur in Aves (figure 1.11), the variation thought to have been caused by replication error. It is generally accepted that mtDNA is maternally inherited, though mitochondrial heteroplasmy is widely debated (McVean, 2001; Kvist, Martens, Nazarenko & Orell, 2003). The implications of this debate are of no concern in the current study due to the use of mtDNA to perform species classification.

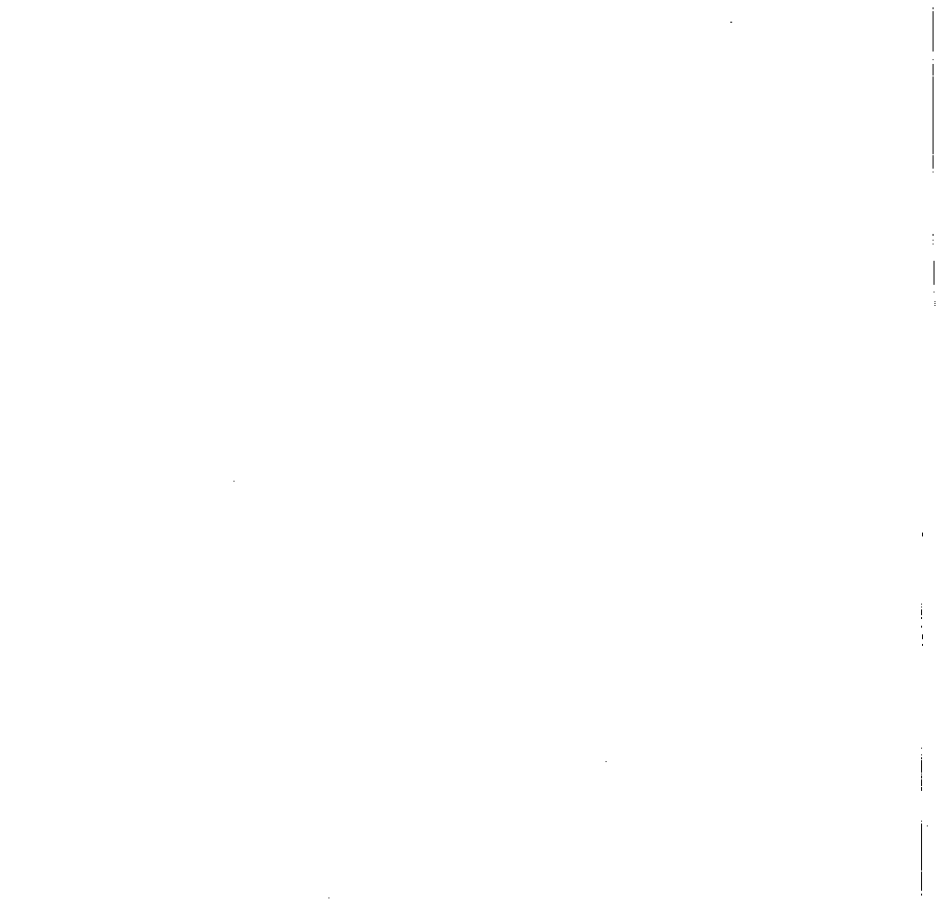


Fig. 1.10: Standard vertebrate mitochondrial genome schematic taken from Pereira (2000b).

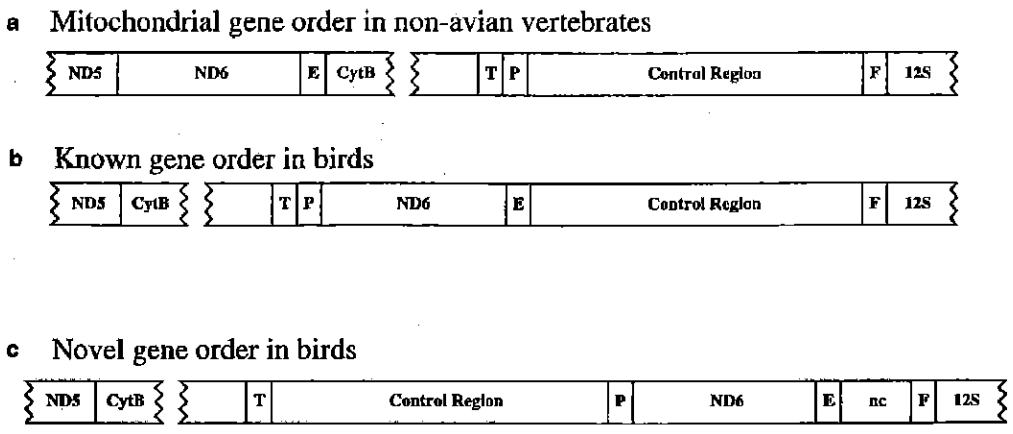


Fig. 1.11: Non-avian and avian gene orders taken from Mindell, Sorenson & Dimcheff, 1998.

The two strands of mtDNA, known as the light (L) strand and heavy (H) strand are named according to the presence of pyrimidines (thymine and cytosine) and purines (adenine and guanine) respectively. Purines contain an additional ring not found in pyrimidines which increases the molar mass of the compound. The L strand runs in the 5' – 3' direction and primers are commonly labelled with 'L' and 'H' to show their direction.

A somatic cell contains one copy of nDNA but numerous copies of haploid mtDNA. The number of mitochondria present within a cell varies depending on cell type and 10-15 copies of the genome are found within each mitochondrion (Linacre, 2009). This high copy number improves the likelihood of DNA isolation (even from degraded substrates) as well as polymerase chain reaction (PCR) amplification and the success of subsequent sequencing. Degradation of mtDNA occurs at a much slower rate than nDNA, thought to be due to the molecules circular structure making it less vulnerable to exonucleases attaching to free ends of the DNA (Seutin, White & Boag, 1990). Mitochondria also have a protein coat which helps prevent bacterial degradation of the organelle making mtDNA much more beneficial than nDNA when working with old or degraded samples.

The rate of mtDNA evolution is thought to be up to 10 times greater than that of nDNA (Brown, George Jr, & Wilson, 1979) due to the absence of the specialised correction mechanism for copy errors which is present in nDNA. The general rule of 2% mitochondrial sequence divergence per million years (Brown et al., 1979) has come under contest as the rate appears to vary between species (Klicka & Zink, 1997; Lovette, 2004). Genome evolution usually results from base substitutions as large insertions and deletions (indels) may alter protein structure. Substitutions are most commonly transitions (purine to purine or pyrimidine to pyrimidine) rather than transversions (purine to pyrimidine or pyrimidine to purine) which are observed less frequently and are therefore viewed as more informative base changes. See figure 1.12 for a diagrammatic representation of this DNA substitution. Protein coding regions often exhibit silent mutations in which substitutions do not alter the amino acid code (Kessler & Avise, 1985).

Fig. 1.12: Transition/Transversion diagram translated from Pereira (2000a).

1.3.2 Evidence of small populations within mitochondrial DNA

MtDNA is well suited for evolutionary studies due to its rapid evolution and lack of recombination (Avise et al., 1987). Species derived from a small founder population or which have undergone a population bottleneck usually contain very little mitochondrial variation (Blondel, 2000; Jamieson, 2009; O'Brien, 1994). This lack of diversity is due to a loss of polymorphisms through inbreeding as the population size decreases (such as is evident in the cheetah population, demonstrated by Menotti-Raymond & O'Brien, 1993).

Because the Trinidad Piping guan is an endemic island species, the original founder population may have had an effect on the number of mitochondrial haplotypes which are seen in the current population. If only a small number of birds remained on the island, the population may be seen to originate from a single female line in future generations due to a loss of haplotypes through inbreeding. Similary, if a large founder population declines through hunting and deforestation, this population bottleneck produces the same trend of haplotype loss. Issues of inbreeding are then presented and genetically important individuals may be removed from the population. This could therefore pose a threat to the species survival through issues such as decreased disease resistance (Spielman, Brook, Briscoe & Frankham, 2004). It would be expected from the trends discussed that the current population of Trinidad Piping guan continues very few variant haplotypes.

1.4 Aims of the investigation

Genetic samples from the island population of the Trinidad Piping guan have never been obtained for genetic study. Previous genetic research of the species (phylogenetic assessments) has been conducted using blood samples taken from a bird held at a private aviary in Mexico. It is possible that the phylogeny created using this individual may not have represented genetic material present in the remaining population of island birds. Samples obtained in the current study can therefore be used to re-evaluate the phylogeny for support of the previous assessment and ensure that sequence data was representative of the species. The use of data which are not archetypal of the species may result in an incorrect branching pattern and mislead interpretation of evolutionary history. An estimation of genetic diversity through the examination of mitochondrial haplotypes can also be performed. These intra-species comparisons have the potential to identify haplotype diversity within the remaining population as well as between island and captive populations.

Sequence data obtained can also be used to identify species markers for rapid identification of field samples. This may be of use in future ecological and conservation studies with respect to population estimates and the identification of areas of habitation. Due to the elusive nature of the species and the desire to avoid stress in sample collection, methods were required which would be suitable for the recovery of genetic material and realistic with regards to collection. By using samples similar to those that may be found in field and forensic investigations, this also ensures the applicability of the methods to future studies. The research aims of the investigation were therefore:

1. To identify suitable non-invasive techniques for the recovery of genetic material to minimise disruption to the species.

2. To investigate genetic diversity within the free-living population and observe potential haplotype variation between the wild birds and captive populations.

3. To obtain sequence data from a pure bred island bird to support the phylogeny of Piping guans and placement of the Trinidad Piping guan.

4. To establish a method of species identification applicable for use on remotely collected or forensic samples to assist field studies and the identification of hunting kills.

Chapter 2

General Methodologies and Materials

2.1 Introduction to general procedures

To investigate all aims of the current study, samples were required from individuals of the remaining Trinidad Piping guan population for the extraction of genetic material. It was necessary that samples be viable for high quality DNA extraction to provide sound reference material for each area of investigation. Successful DNA extractions were used throughout the course of study for the amplification of mtDNA fragments to investigate intra and inter-species diversity and establish a method of species detection. The standard methods of DNA extraction, amplification and fragment separation used throughout the text are described below. A full description of successful primers for mtDNA amplification found by experimentation is provided with all methodological specific primers and conditions described per chapter as appropriate.

2.2 Biological samples used for DNA extraction

With respect to the species of study, six moulted feathers were obtained from the captive Trinidad Piping guan held at the Port of Spain Zoological Garden in Trinidad, used as the reference material for the current study. As with Grau, Pereira, Silveira, Höfling & Wajntal, (2005) who obtained blood samples of a Trinidad Piping guan held at a private aviary in Mexico, samples were not voucher specimens. The samples were received on CITES licence number 1053 in addition to permits obtained from the Trinidad Forestry Division. All flight feathers were stored at -17^{o}C until project commencement.

Over the course of the study, three further consignments of field samples thought to have originated from Trinidad Piping guan were opportunistically collected by field researchers within known habitat of the free-living population. Two feather samples were received, the first being the quill tip of a flight feather recovered from the forest floor of Morne Bleu and the second a full contour

feather obtained from the Grande Riviere. A CITES permit (1103) and export licence from the Trinidad Forestry Division were obtained for these samples allowing for up to six feathers to be exported within the time-scale of the licences. The final suspected Trinidad Piping guan consignment originated from an egg which had fallen from a nest in Trinidad. From this egg, liver tissue, developing feathers, eggshell membrane and the egg soup contents were received. CITES permit 1116 allowed for two consignments of up to 20g of material to be exported. Due to the nature of the egg samples, a permit (POAO/2009/249) was also obtained from Defra who required all samples to be heat treated at 56^{o}C for 30mins prior to export.

2.2.1 Further taxa and permits obtained during the course of study

In addition to the target species, samples from other species were also obtained to perform comparative tests and phylogenetic assignment. Feathers were obtained from museum samples of Gray's Piping guan (*Pipile cumanensis grayi)* and Bare-faced curassow (*Crax fasciolata)* from the Museu de Zoologia da Universidade de São Paulo on Defra import authorisation POAO/2009/050. No CITES permits were required as these species are not registered on the IUCN Red List. Moulted feather samples were also obtained from the Red-billed curassow (*Crax blumenbachii*) and Black vulture (*Coragyps atratus*) held at Chester Zoological Gardens. Feathers from the domestic chicken (*Gallus gallus domesticus*) were obtained from a small holding (Personal communication, June Bayley, 7 April, 2008). Samples obtained from species outside of the *Pipile* family were required to test the methods of species detection devised in the current study to ensure a positive result was not produced for non-target species. For some of the taxa required in the current study, samples could not be obtained and therefore sequence data from previous studies was recovered from GenBank for comparative analysis. The species for which data was recovered and the corresponding accession numbers can be found in Table 2.1.

Table 2.1: Accession numbers of GenBank data used in the current study.

Species	Accession number	
	ND2	Cyt *b*
Pipile pipile	AY367094	AY367106
	AY367100	-
Pipile cumanensis grayi	-	AY659797
Pipile cumanensis	AY367093	AY659798
	AY367099	AY367105
Pipile cujubi	AY367092	AY659799
	AY367098	AY367104
Pipile jacutinga	AY140744	AF165476
Aburria aburri	AY140740	AF165466
Penelopina nigra	AY140743	AF165475
Chamaepetes goudotii	AY140741	AF165467
Chamaepetes unicolor	-	AY659796
Ortalis poliocephala	-	AY659784
Ortalis pantanalensis	-	AY659783
Ortalis guttala	-	AY659782
Ortalis ruficauda	-	AY659781
Ortalis garrula	-	AY659780
Ortalis leucogastra	-	AY659779
Ortalis motmot	-	AY659778
Ortalis canicollis	AY140746	AF165472
Oreophasis derbianus	AY140745	AF165471
Mitu tomentosa	AY141938	AY141928
Mitu salvini	AY141937	AY141927
Mitu tuberosa	AY140748	AF165469
Mitu mitu	AY141936	AY141926
Nothocrax urumutum	AY140749	AF165470
Crax pauxi	-	AF068190
Crax blumenbachii	AY140747	-
Crax rubra	AY141935	AY141925
Crax globulosa	AY141934	AY141924
Crax fasciolata	AY141933	AY141923
Crax daubentoni	AY141932	AY141922
Crax alector	AY141931	AY141921
Crax alberti	AY141930	-
Pauxi gilliardi	-	AY659788
Pauxi unicornis	AY141939	AY141929
Pauxi pauxi	AY140750	AF165473

2.3 DNA extraction

DNA was extracted from 5-10mm of the inferior umbilicus of feather samples using Qiagen DNeasy blood and tissue kit with incubation performed in a shaking water bath for two hours. A user-developed protocol was followed in which the only variation from the manufacturers' protocol was the removal of the un-lysed quill tip from the extraction before centrifugation in the DNeasy mini spin column. With respect to the samples received from the egg; DNA extraction was performed on a $5mm^3$ section of the liver, 10 developing feathers and $1.5cm^2$ of the chorioallantoic membrane prepared with sterile equipment. DNA extraction was performed in a separate laboratory to the previous samples to reduce the possibility of contamination.

Extractions from other species were performed in a separate area of the laboratory and employed different equipment to that used for PCR preparation. Disposable pipette tips were used in all instances of reagent transfer and pipettes were sterilised under UV light between each DNA extraction. Sample preparation kits including scissors, tweezers and scalpels were all sterilised by autoclave at 121^oC with a separate kit used for each sample. The rationale of the biological samples obtained in the current study is discussed in chapter 3.

2.4 Mitochondrial genome regions sequenced in the current study

The focus of the current study was placed on the protein coding genes Cytochrome *b* (cyt *b*) and NADH-dehydrogenase subunit II (ND2) of which whole gene sequencing was performed. Also sequenced were the truncating tRNA's; tRNA Thr and tRNA Pro as well as the WANCY cluster containing tRNA Trp, tRNA Ala, tRNA Asn, tRNA Cys and tRNA Tyr. In addition, the non-coding D-loop was partially sequenced.

Due to the little genetic research which has previously been conducted for the species, the process of identifying successful amplification primers required research by experimentation. Many standard universal combinations were unsuccessful and as full gene sequencing was required, overlapping primers had to be located within previously published literature. Combining primers from

multiple publications created problems of compatibility and therefore PCR conditions were evaluated by experimentation for each newly combined set.

2.4.1 DNA amplification

All PCR reactions in the current study were prepared in a UV sterilization cabinet with equipment used solely for PCR preparation. The cabinet and equipment underwent 20mins of UV exposure (260nm) prior and subsequent to each preparation. The DNA template was added to the reactions within a separate cabinet to avoid DNA contamination of the PCR preparation area.

Amplification was performed using Amersham Biosciences PuReTaq Ready-To-Go 0.2ml PCR beads containing BSA, 200µM of each dNTP (dATP, aCTP, dGTP, dTTP) 10mM Tris-HCl, 50mM KCl and 1.5mM $MgCl_2$ in a 25µl reaction with 25pmol of the primer pair, 19µl of sterile distilled water and 5µl of the unpurified DNA extraction performed in a Techne Flexigene thermal cycler.

2.4.2 ND2 Universal Primers

Initially, avian universal primers were used to amplify the ND2 gene. These primers have proven successful on a wide variety of avian species and would therefore provide sequencing from which primers specific to the Trinidad Piping guan could be designed. Primers L5216, H5766, L5758 and H6313 (Sorenson, 2003) were used to amplify the ND2 gene in two halves. Upon sequencing with the amplification primers it was found that the two sequences did not overlap due to a loss of sequence data during the cycle sequencing process. In order to obtain the sequence between these amplicons, primers were designed within the final section of the first amplicon (Lc5568) and the beginning of the second amplicon (Hc5608). When sequenced, the ends overlapped with the initial sequencing allowing the two halves of the gene to be combined. Data for the ND2 gene was therefore obtained minus the first 8 bases lost in sequencing. Cycling parameters for these three primer sets were an initial denaturation of 5mins at 94^{o}C followed by 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 2mins at 72^{o}C with a final extension of 30mins at 72^{o}C.

2.4.3 Degenerate bases

Some primers used to amplify the ND2 gene contain degenerate bases. These bases are often used in universal primer design in order to accommodate variation seen within genetic sequences between species. Because of the divergence which occurs between distantly related species, degenerate bases are incorporated in order to overcome discrepancies. By analysing the Single Nucleotide Polymorphisms (SNPs) within the alignment, bases can be chosen which will enable the extension of the primer in spite of mutation (see table 2.2).

Table 2.2: Description of degenerate bases.

Degenerate Base	Coding definition	Codes for
W	Weak interaction	A or T
S	Strong interaction	G or C
M	aMino	A or C
K	Keto	G or T
R	puRine	G or A
Y	pYrimidine	T or C
B	not A	T or G or C
D	not C	A or T or G
H	not G	A or T or C
V	not T	A or G or C
N	aNy base	A or G or T or C

2.4.4 Cytochrome **b** *Universal primers*

Amplification of the Cyt *b* gene presented some problems as primers were either unsuccessful or did not amplify large enough regions in order for sequencing to be combined to create whole gene sequence data. To overcome this problem, primers were taken from a number of publications to amplify the gene in three overlapping sections. Initial sequencing was conducted using the universal primer L14841 taken from Kocher et al., (1989) combined with H15670 from Kimball, Braun, Zwartjes, Crowe & Ligon, (1999) under conditions of 5min at 94^{o}C followed by 35 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 2min at 72^{o}C with a final extension of 30min at 72^{o}C. From this initial amplification a reverse primer was designed (CytbD) within the beginning of the sequence data to be

combined with ND5 from Thommasen, Thomson, Shutler & Kirby, (1989) and overlap L14990 from Kornegay, Kocher, Williams & Wilson, (1993). Amplification of the ND5/CytbD primer pair was therefore performed with an initial denaturation at 94^{o}C for 5min followed by 35 cycles of 30sec at 94^{o}C, 30sec at 60^{o}C and 1min at 72^{o}C and a final extension of 5min at 72^{o}C.

The forward primer L14990 was combined with the previous H15670 from Kimball et al., (1999) for amplification of the Trinidad Piping guan samples with cycle parameters of 5min at 94^{o}C followed by 40 cycles of 1min at 94^{o}C, 1 min at 50^{o}C and 2min at 72^{o}C with a final extension of 20min at 72^{o}C. This reverse primer was however unsuccessful when amplifying from *Pipile cumanensis grayi* and an alternate primer of H15696 from Kornegay et al., (1993) was used for this amplification with cycle parameters altered to 5min at 94^{o}C and 40 cycles of 1min at 94^{o}C, 45sec at 50^{o}C and 2min at 72^{o}C and a final extension of 10min at 72^{o}C. For the final stage of cyt *b,* amplification primers L15311 and H16065 (Kimball et al., 1999) were used which underwent conditions of 5min at 94^{o}C and 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 1min at 72^{o}C with a final extension of 5min at 72^{o}C.

2.4.5 Principles of amplification/sequencing primer design

Primer design was conducted following standard principles including the creation of primers between 18-24 bases in length containing a GC content of 40-60% (Dieffenbach, Lowe & Dveksler, 1993). Because of the higher bond stability seen between bases G and C (due to binding by 3 hydrogen bonds rather than 2 as seen between A and T) GC content increases the melting temperature of a primer. Melting temperature (T_m) can be calculated with the simple formula 'Itakura's imperial rule':

$$T_m = 2\,(A + T) + 4\,(G + C)$$

This generates the 'Wallace temperature' but is only valid for use on primers which are between 14 – 20 bases. A more accurate quantification of T_m includes the salt concentration and oligonucleotide length using the formula:

$$T_m = 81.5^{o} + 16.6(\log[M^{+}]) + 0.41(\%G+C) – (500/N)$$

Where *M*+ is the concentration of KCl and *N* is the number of nucleotides in the primer (Stephenson, 2003). When designing a primer pair, each primer must have a similar T_m to ensure efficiency.

The annealing temperature (T_a) is generally 3-5^{o}C below the T_m which if set too low will result in lack of primer specificity and too high will reduce hybridization and therefore PCR product. Primers must also avoid areas of secondary structure and stretches of base runs and repetition as well as ensuring that a primer dimer is not created in which primers anneal to each other rather than the target sequence (Applied biosystems, n.d.).

All designed primers in the current study were selected by eye, assessing a region of 100bp in which the primer was required. When a suitable region was identified and forward and reverse primers designed, the T_m was calculated with the latter of the previously mentioned formulae. If the difference between T_m was too great (>8^{o}C) between each primer pair; the base sequences were re-assessed to find a more compatible match. Following the aforementioned principles, species specific primers were designed from the sequence information obtained from the Trinidad Piping guan mitochondrial genome which is discussed in detail in chapter 5.

2.4.6 Primers used during the course of the study

Sequencing was obtained in regions from ND2 to COI and Cyt *b* to D-loop including all tRNA clusters; sequence data for these regions can be found in Appendices I. Figure 2.1 shows a representation of the standard avian mitochondrial genome to which the Trinidad Piping guan conforms, including the approximate positions of the L strand primers. Table 2.3 displays all successful

amplification/sequencing primers used in the current study. For all primers used throughout the course of the study see Appendices II (primer references in Appendices II can be found within the current chapter references).

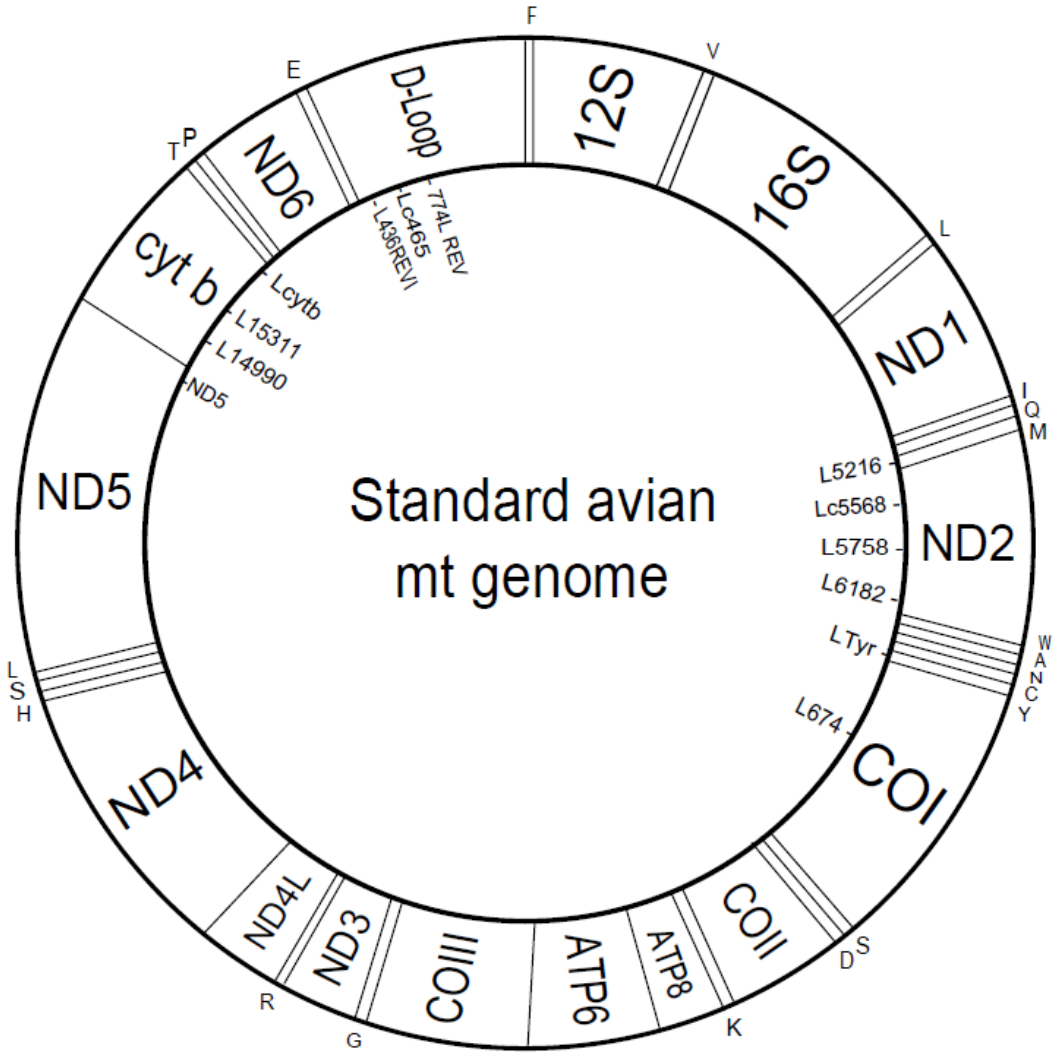


Fig. 2.1: Representation of the standard avian genome displaying the regions in which the forward primers used in the current study lie, redesigned from the format of Sorenson (2003).

Table 2.3: Primers used to amplify and sequence regions of the Trinidad Piping guan mitochondrial genome taken from previous publications or designed in the present study.

Mt locus	L Suffix	L strand primer 5'- 3'	H suffix	H strand primer 5'- 3'	Origin
ND2	**L5216**	GGCCCATACCCCGRAAATG	**H5766**	RGAKGAGAARGCYAGGATYTTKCG	Sorenson (2003)
ND2	**L5758**	GGNGGNTGAATRGGNYTNAAYC ARAC	**H6313**	ACTCTTRTTTAAGGCTTTGAAGGC	Sorenson (2003)
ND2	**Lc5568**	AGGATCGCACCTAACCACTGCC	**Hc5608**	GTAGGCTGAGTTTGGGGCTA	Designed
Cyt *b*	**ND5**	TAGCTAGGATCTTTCGCCCT	**CytbD**	TGTAGGTTGCGGATTAGCCAGC	Thomassen (1989) & Designed
Cyt *b*	**L14990**	AAAAAGCTTCCATCCAACATCTC AGCATGATGAAA	**H15696**	AATAGGAAGTATCATTCGGGTTTG ATG	Kornegay (1993)
Cyt *b*	**L14990**	AAAAAGCTTCCATCCAACATCTC AGCATGATGAAA	**H15670**	GGGTTACTAGTGGGTTTGC	Kornegay (1993) & Kimball (1999)
Cyt *b*	**L15311**	CTCCCATGAGGCCAAATATC	**H16065**	TTCAGTTTTTGGTTTACAAGAC	Kimball (1999)
ND6	**Lcytb**	CATGAGTGGGCAGCCAACCAGT	**Hdloop**	GCTAGAAAGATGCATGTCCGTC	Designed
D-loop	**L436REVI**	CCTCACGAGAAAYCAGCAAC	**H774**	CCATATACGCCAACCGTCTC	Sorenson (1999)
D-loop	**Lc465**	GCGCCTCTGGTTCCTATATCA	**Hc839**	AAGCATTCACTAAATAGCACCA	Designed
D-loop	**774LREV**	GAGACGGTTGGCGTATATGG	**H1251 REVI**	TCTTGGCATCTTCAGTGCCRTGC	Sorenson (1999)
WANCY	**L6182**	TGGTATCTCCGTAAAACACC	**H6735**	GCCTACTATGCCTGCTCATGTA	Designed
COI	**LTyr**	TGTAAAAAGGWCTACAGCCTAAC GC	**H763**	GCGTAGTATGCTACTACGTGG	Pereira & Baker (2004) & Designed
COI	**L674**	GGAGACCCAGTTCTATATC	**H8205**	GGTTCGATTCCTTCCTTTCTTG	Pereira & Baker (2004) & Designed

All primers designed from universal sequencing conducted in the current study except 'Hdloop' which was designed using GenBank Pipile pipile *data AY145320.*

2.5 Electrophoresis

All PCR amplifications were separated on 2% agarose gels pre-stained with Biotium GelRed™. 2g agarose was dissolved in 98ml ddH_2O by heating and when cooled, 2ml 50x TAE and 10µl GelRed™ were added. 50x TAE buffer was made by dissolving 242g Trizma base in 100ml 0.5M EDTA and adding 57.1ml glacial acetic acid. The volume was then made up to 1 litre with distilled water, buffered to pH8 and sterilized by autoclaving. The wells of the gel were placed at the cathode and 2x TAE running buffer added. 10µl of Norgen Biotek Corporation PCRSizer 100bp DNA ladder of known concentrations (see figure 2.2) was added to the first well of the gel and DNA amplicons loaded in the remaining 15 wells which contained 10µl of the DNA amplicon and 2µl 6X blue loading dye (which migrates at approximately 300bp). The gels were run at a constant 100V and visualisation was achieved with ChemiDoc™ XRS UV transilluminator. Images were captured using BioRad Molecular Imager® and formatted with Quantity One 1D analysis software.

Fig. 2.2: Norgen Biotek Corporation PCRSizer 100bp DNA ladder. Image from Norgen Biotek Corporation (n.d.)

2.6 Cycle sequencing and data handling

All sequencing reactions were performed by MWG Eurofins. Amplification primers were used for all sequencing reactions at a concentration of 2pmol/µl with DNA template from the original amplification quantified by MWG Eurofins and diluted to a sufficient concentration for sequencing. Results were obtained in FASTA format and an electropherogram of sequencing data received (an example can be seen in Appendices III).

Any discrepancy between forward and reverse sequence was verified by eye using the electropherogram provided and only double stranded sequencing with a phred score of ≥30 (99.9% accuracy) was accepted. Sequences were aligned using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997) and converted to postscript subsequently viewed using GSview 4.9 (Lang, 2007) and exported to pdf. All phylogenetic analysis of data was carried out using jModelTest (Posada, 2008) and MEGA4 (Tamura, Dudley, Nei & Kumar, 2007) using the FASTA file created by ClustalX alignment. As the sequence data generated in the current study has not yet been published, the data has not received an accession number for identification. When referring to sequence data generated in the present study, a coding system was devised to identify the species, sample and gene, for example:

Pp_C.F.2008/ND2

Species	Pp = *Pipile pipile*	Ppg = *Pipile cumanensis grayi*
Sample source	C = Captive	V = Voucher W = Wild
Sample type	F = Feather	T = Tissue
Date	Year received	
Gene	Gene sequence	

Chapter 3

Review and establishment of avian sampling methodologies for use in the current investigation

Introduction

3.1 Analysis of sampling methodologies for the species of study

In avian genetic studies there are many issues surrounding sampling methodologies which may influence selection. The viability of a method must be considered for each species individually as an invasive method involving capture which is suitable for common species may not be plausible when studying elusive and/or endangered species.

Considerations must be taken into account including the expertise of the sample collection team, the resources which are available, the chance of capture and the issues surrounding it such as capture stress and nest desertion (Matson, Tieleman & Klasing, 2006; Colwell, Grattio, Oring & Fivizzani, 1988; Criscuolo, 2001). If methods such as mist nets are used, the time taken to remove birds from nets can influence the level of stress (Romero & Romero, 2002) and presents dangers of predation of captured birds (Brooks, 2000). If invasive methodologies are deemed unsuitable for the species of study, non-invasive techniques can be undertaken. This approach is not without its limitations, mainly due to the condition of the material obtained, which must also be considered for the boundaries of an investigation. The selected method must therefore suit not only the species of study but also the analysis which is to be conducted. For example, remotely collected samples which have remained in the field for many weeks may not be suitable for the study of nDNA due to degradation issues. The sample obtained must therefore be capable of providing viable DNA for analysis to avoid re-sampling and sustain the cost of an investigation. The possible limitations of certain materials with respect to DNA quality should also be understood before conducting genetic analysis.

3.2 Museum sampling

If the species of study is extinct or samples difficult to obtain, researchers can obtain feather samples from museum specimens. Although tissue from footpads can be used, this represents a much more destructive process which is less likely to be approved. Footpad tissue can only be used by a few study groups before being exhausted and its removal could limit the scope of adaptation studies investigating toepad morphology (Payne & Sorenson, 2002).

Museum samples gained attention in 1984 when Higuchi, Bowman, Freiberger, Rider & Wilson successfully extracted and sequenced DNA from the extinct quagga (*Equus quagga quagga)*. Although extractions were generally of low quantity and molecular weight, a new avenue of genetic research was opened. The quantity of DNA extracted was 5-20% of that which would be expected from a fresh sample (Houde & Braun, 1988) but with the advent of PCR (Saiki et al., 1988) it became possible to amplify limited amounts of ancient DNA. Pääbo (1989) utilised PCR to study the modifications seen in archive DNA sequences that have been caused by oxidative damage. It was found that molecular size did not correlate with the age of the sample, providing evidence that the treatment of the specimen with regards to preservation is a more important factor. This observation is in agreement with Hogan et al., (2007) who demonstrated that feather quality is more important than feather type i.e. quality is imperative.

3.2.1 Maintaining museum collections

Although the use of alternative samples for genetic investigation is a positive avenue for conservation biologists and the study of endangered or extinct species, the resources of museums must be renewed to maintain collections for future studies. Winker, Fall, Klicka, Parmelee & Tordoff (1991) argued a case for the continued collection of samples in order to maintain museum resources and criticised the restrictions placed on scientists in comparison to that of hunters claiming that only 0.00011% of avian mortality is caused directly by scientific and educational research. Edwards, Birks, Brumfield & Hanner (2005) indicated

that the demand for what has been called GRCs (genetic resources collections) is at an all time high. Samples must therefore be replaced during fieldwork whilst still preserving natural populations by obtaining opportunistic tissue samples or non-invasive samples. This is also the agenda of The Frozen Ark project who promote the preservation of samples from species threatened by extinction (The Frozen Ark, n.d.). The advantages of collecting feathers for future study have been highlighted by Smith (2003) who remarked that around 1.2 million songbirds are banded each year, representing a large untapped resource of feather sample collection, previously suggested by Morin et al., (1994) who believed that this should become routine practice. It has been suggested that ancient feather samples could be returned to the museum and replaced in the specimen after the quill tip is removed, highlighting the difference between 'destructive sampling' and 'value added sampling' (Payne & Sorenson, 2002). Collections such as that housed in the Burke Museum in Washington DC play a large role in phylogenetic investigations with around 60-70% of loans being used for this purpose. Edwards et al., (2005) also point out that this will become a huge resource for large projects such as DNA barcoding, generating a database of species-specific genetic sequences. Because Trinidad Piping guan materials were not taken from a museum specimen, it is important that samples obtained undergo appropriate storage and treatment to preserve material for future study. In order to generate public availability of samples, purified DNA extractions could be donated to The Frozen Ark for use by other study groups.

3.2.2 Extraction of DNA from museum samples

In 1993 Leeton, Christidis & Westerman reported a protocol for extraction of DNA from the feather tips of museum bird skins claiming that this type of sample would help prevent contamination, especially when taken from the wing where interference during preparation is minimal. Unfortunately, as these feathers are normally used for measurements, obtaining wing feathers for genetic study is rare. Leeton et al., (1993) also found no correlation between the quantity of DNA recovered and the age of the sample - in concurrence with Pääbo (1989) and

Hogan et al., (2007). Hofreiter, Serre, Poinar, Kuch & Pääbo (2001) discussed ancient DNA degradation in greater detail and recommended the use of multicopy (mitochondrial and chloroplast) DNA as degradation rate is reduced.
Payne & Sorenson (2002) report a decrease in the length of PCR products obtained as the age of the sample increases, though in this instance the quality of the sample was not considered. This result may however be explained by the length of time that exonucleases have to degrade the DNA, which if not well preserved will occur at a much faster rate (Seutin, White & Boag, 1990). DNA extraction may therefore be successful in samples which differ greatly in age but PCR success will be reflected by the size of the fragment which is being amplified. Due to the recent analysis of DNA from feather barbs of extinct taxa, this minimally destructive process may provide opportunity for future genetic studies (Rawlence et al., 2011).

3.2.3 Trinidad Piping guan museum samples

Due in part to the elusive nature and disputed classification of the Trinidad Piping guan, the species has been the focus of very few studies and no genetic research has been conducted on voucher/museum specimens. In the current study, only two publications were located which described museum specimens (Vaurie, 1967; Lopes 2009) with three specimens mentioned in each paper. Vaurie (1967) describes three specimens (2 male, 1 female) from the American Museum of Natural History (AMNH) and Lopes (2009) 3 male specimens, 2 from AMNH and one from the Sabah Museum (SMM) (see table 3.1). In Vaurie (1967) these Trinidad samples are classified as *Pipile pipile pipile* and in Lopes (2009), *Aburria pipile*. It would appear that these articles describe six separate Trinidad Piping guan specimens due to the difference in sex of the Caparo sample at AMNH.

Table 3.1 Museum specimens described in Vaurie (1967) & Lopes (2009)

Author	Museum	Sex	Origin	Location
Vaurie	AMNH	M	Trinidad	Platinal Valley
Vaurie	AMNH	M	Trinidad	Princetown
Vaurie	AMNH	F	Trinidad	Caparo
Lopes	AMNH	M	Trinidad	Caparo
Lopes	AMNH	M	Trinidad	Princess Town
Lopes	SMM	M	Trinidad	Aripo

Although museum samples were available, the aims of the current investigation focused on the analysis of mitochondrial haplotypes within the remaining population with respect to genetic identification and the verification of previous data as representative of island birds. In this instance, using museum samples to create a method of species identification may prove ineffective due to the dramatic population decline of the species. Mitochondrial haplotypes found within museum samples may have been removed from the current population and therefore detection methods established using this data may not be applicable to birds currently found in Trinidad. As full gene sequencing was required to locate species defining regions, the use of field samples also provided a greater likelihood of extracting and sequencing significant portions of genes in a single reaction for analysis.

3.3 The importance of sampling methodology selection in endangered species research

Sampling methodology selection can be imperative to the success of extraction of genetic material which is required for investigation. Selecting an inappropriate method can result in the collection of non-viable samples, insufficient DNA extraction or unnecessary disruption to the species. This decision is heightened when working with endangered species as the opportunity for collection may be limited by ecological and permitting issues. The collection of materials must therefore suit the aims of the study and be feasible for personnel collecting

biological material in the field. Collection, storage and transportation must all be taken into account and viable methodologies appropriate to the material collected must be established to ensure that samples meet acceptable health and safety regulations and are preserved in transit.

The most common sample types obtained for avian DNA analysis fall into the categories of tissue, blood, faecal and feathers. Methodologies take an invasive or non-invasive approach to sampling depending if the species of study is to be captured. Tissue and blood samples are normally classed as invasive and faecal samples non-invasive. Feather samples however, can be collected either invasively by plucking or non-invasively by the collection of naturally shed feathers. When sampling from a living specimen, feathers are therefore more adaptable to a range of investigations and sampling opportunities may occur more readily than if the researcher restricts their methodology to, for example, blood collection. However, if the species of study is extinct or no personnel are available in the field to collect biological material then genetic resources can be obtained from museum specimens through invasive/destructive sampling (for a full description of terminology see table 3.2).

Feather sampling techniques are of great importance when working with endangered avian species (Piggott & Taylor; 2003). The interruption of daily routines and induced stress when conducting invasive sampling may have serious implications. Handling some birds may cause nest abandonment and blood collection can occasionally result in fatalities (Hoysak & Weatherhead, 1991). The stress caused by invasive sampling could therefore put the bird at risk when subsequently released and may cause it to vacate the area it normally inhabits. This is especially problematic should it result in the bird leaving protected habitat, such as that in which the Trinidad Piping guan resides. In this respect, non-invasive techniques remove the necessity to capture, handle, or even directly observe birds and also allow for opportunistic collection. In addition, taking samples from captive specimens will often be reliant on non-invasive techniques as keepers will want to avoid unnecessary stress to a bird, especially when the species is endangered or forms part of a breeding program.

Table 3.2: Summary of the terminology associated with sample collection and examples for each methodology.

	Terminology	Sample type
Sample collection without capture	1. Non-invasive sampling 2. Non-invasive Genetic Sampling (NGS)	Moulted feathers, nest materials, guano
Sample collection with capture	3. Invasive sampling 4. Non-destructive sampling 5. Destructive sampling*	Blood, tissue, plucked feathers
Museum sampling	6. Destructive sampling 7. Value added sampling	Plucked feathers, tissue

* In papers which involved sacrifice of a bird.

These terms were first described in: 1. Morin, Messier & Woodruff, (1994); 2. Roon, Thomas, Kendall & Waits, (2004); 3. Taberlet & Bouvet, (1991); 4. Marsden & May, (1984); 5. Smith & Zimmerman, (1976); 6. & 7. Payne & Sorenson, (2002).

3.3.1 Invasive sampling

Tissue samples can be obtained invasively when a bird is to be euthanized for the purpose of the study (a rare practise in modern research and not viable for the study of endangered species) or from animals which have been found dead. This can occur through natural causes or slaughter and includes tissues obtained in forensic casework. Another example of invasive sampling is that taken from museum samples which also comes under the title of 'destructive sampling' as it is not invasive to the species in question. Decomposition and degradation can affect the success of DNA methodologies, therefore recently deceased birds or well preserved tissues will provide a higher quality of DNA. These effects are more apparent when working with nDNA due to the lower copy number and higher rate of degradation than that which is seen in mtDNA. However, if the full carcass remains, DNA can be extracted from alternative samples such as bone which undergo a slower rate of degradation and therefore preserve genetic material.

In the case of the Trinidad Piping guan, tissue samples were not deemed to be a viable resource due to the species Critically Endangered status and little chance of finding an individual which has died of natural causes. However, one purpose of the current research was to establish a method of species identification and as such, the method established must also be applicable for use on tissue samples should an opportunity arise.

Direct blood sampling can only be performed if the sampling methodology includes capture. However, if the species is the focus of study in another research project adopting invasive methodologies (such as ringing or fitting radio collars) this is a good opportunity to remove samples such as blood for future study and prevent re-capture. Although researchers in Trinidad have attempted to capture individuals of the Trinidad Piping guan for study, this has so far been unsuccessful. Should an individual have been caught within the time frame of the investigation, blood sampling could have been performed by a veterinarian for use in the current study. This sample would therefore provide a sound source of DNA which has not undergone degradation or contamination and could be

used for nDNA studies if required. Due to the minimal amount of material required for DNA extraction, this blood sample could be separated into a number of aliquots after collection. These samples could then be kept frozen at UWI in Trinidad and sent on request to future research groups for study. A single blood sample could therefore provide enough quality genetic material for years of future study. In addition, avian buccal swabs can be taken for genetic analysis and have been found to be successful in genotyping (Yannic et al., 2011). As the number of swabs which can be taken is not limited in the same manner as blood collection, multiple buccal swabs could be conducted (within reason) and the material collected stored for future study.

With respect to invasive feather sampling, this methodology also requires capture of the species of study and specialist training of personnel. The Identification of mature feathers is necessary as a growing feather will bleed if plucked and their removal should be avoided. Great care must be taken during this process to avoid breakage of the quill as any remainder left in the skin will prevent the generation of a new feather (Marsden & May, 1984). Once more, this method would only have been possible through opportunistic collection of materials via other research projects. As many of the health studies to be conducted on the species were working in collaboration with veterinarians, suitably trained personnel were available in the current study for the collection of blood and feather samples should the opportunity have arisen.

3.3.2 Non-invasive sampling

Faecal matter can be collected opportunistically for use in genetic studies (Zinck, Duffield & Ormsbee, 2004) but requires specialist knowledge to identify samples and appropriate storage conditions to prevent the growth of mould. Problems may also arise in DNA analysis as the quantity of material extracted may be low and contain contamination from consumed plants or animals. It is now known that environmental conditions and the surface from which the faeces were recovered can also have an effect on the extraction of DNA (Oehm, Juen, Nagiller, Neuhauser & Traugott, 2011). Taking into account the observed

behaviour of spending <1% of time on the forest floor (Hayes et al., 2009) the chance of finding guano in an area in which a swab could be taken was limited. This sampling methodology would therefore not have been logistically or practically feasible in the current study.

Feathers are a potentially useful DNA resource as the properties of the feather enables simple storage and transport (Marsden & May, 1984), ease of detection and reasonable persistence in the field (Booms, McCaffery & Schempf, 2008). Though degradation does occur, the keratin structure of the quill helps to protect DNA from damaging environmental conditions. Feathers are durable; therefore if it is suspected that a sample has become contaminated by extraneous DNA or substances which may inhibit PCR, the sample can be washed. In the present study, invasive sampling may have been possible via the collection of blood samples during health studies and non-invasive sampling by the opportunistic collection of moulted feathers by field researchers in Trinidad.

Methods

3.4 Comparison of blood and feather sampling

There is much debate as to which sampling methodology should be used in avian genetic studies to obtain viable DNA and reduce direct stress to the bird. Jamieson (2009) presented the argument that if conducted correctly, the collection of blood may be no more distressing than plucking feathers as stress would peak during the initial capture and handling of the bird. Waite (1990) had previously suggested that the energy expenditure of replacing feathers is greater than that required to replace a blood sample and Hoysak & Weatherhead (1991) claimed that blood sample collection does not affect the health or behaviour of birds which can tolerate being handled.

However, taking blood requires expertise not only in the practical aspect of handling and ensuring the needle does not pass through the vein but also knowledge of how much blood can be safely removed from the bird being sampled (Hoysak & Weatherhead, 1991). Jamieson (2009) also suggests, contrary to popular opinion, that the birds' health with regard to induced stress

should not influence sample selection because of the wealth of information which can be gained from the specimen. Feathers were however recommended for DNA sexing as an alternative for researchers inexperienced with blood collection. In addition, a significant difference in the quantity of nDNA between feathers and whole blood was identified (Jamieson, 2009) and therefore genotyping errors and contamination which may occur with feathers can make them less useful for analysis of nDNA. Conversely, Bayard De Volo, Reynolds, Douglas & Antolin (2008) state that feathers have a high potential as a source of DNA as the feather shaft protects genetic material from environmental conditions which may cause degradation and therefore potential errors in analysis. Harvey, Bonter, Stenzler & Lovette (2006) compared blood and feather samples for their usability with molecular sexing and found the only limitation of feathers was the need for more PCR cycles to obtain sufficient DNA for analysis. Both blood and plucked feather sampling require prior knowledge and expertise and should not be performed as opportunistic samples if the researcher does not have the required training. It is clear that when working with endangered or elusive species, the collection of plucked feathers may not be an option and moulted feathers may provide the most obvious and viable alternative for genetic analysis. In this respect, a decision to undertake non-invasive sampling also eliminates stress to the animal and removes the necessity of trained personnel for sample collection.

It is clear that although some unfavourable consequences may occur when undertaking feather sampling compared to blood, the limitations are more apparent when nDNA is required due to its higher rate of degradation and lower copy number. Viable avian nDNA is more likely to be obtained when using a blood sample due to the presence of nucleated red blood cells. If invasive sampling is undertaken in a study, the genetic analysis to be performed may therefore influence whether blood or feathers are taken from the species.

3.4.1 The viability of invasive sampling to the current study

Blood collection and invasive feather sampling are clearly reliant on capture and in the case of the Trinidad Piping guan, little is known regarding specific feeding or breeding locations. Capture from the free-living population is therefore problematic and the likelihood that a bird would be caught within the time frame of the investigation doubtful. In addition, the storage and transport of blood samples may also have proven impractical and the transfer of bio-hazardous materials inappropriate. To improve the chance of obtaining samples for analysis, the decision was taken to adopt non-invasive feather sampling. The requirement of mtDNA rather than nDNA also lends itself to this approach and minimises problems with exportation of materials.

3.5 Feather samples as a suitable DNA resource

Sandnes (1954) developed a technique for extracting pulp cells from growing feathers in order to examine avian chromosomes and the importance of preventing sacrifice of the bird under study was emphasised by Sasaki, Ikeuchi & Makino (1968). Despite these previous publications, feather sampling was undertaken by few researchers and up until the 1980's sacrifice of the bird under study was common practice. It was not until Marsden and May (1984) continued the development of feather sampling techniques using pulp from the shafts of newly emerging feathers, that the method began to gather pace. Since this publication in 1984, feather sampling has become a well established methodology (Morin et al., 1994; Segelbacher, 2002; Rudnick, Katzner, Bragin & DeWoody, 2007; Bayard De Volo et al., 2008) and is recognised as a fundamental research technique (Piggott & Taylor, 2003; Hogan, Cooke, Burridge & Norman, 2007; Jamieson, 2009; Kerr et al., 2007). The most important advance towards feather sampling as a viable methodology was the development of the polymerase chain reaction (PCR) by Saiki et al., in 1988. In 1991, Taberlet & Bouvet used this technique to study mtDNA lineages of blue tits (*Parus caeruleus*) by extracting DNA from the pulp cells of mature plucked feathers. The result of this study clearly identified the potential of feathers to

provide samples for DNA typing, thereby suggesting for the first time that feathers may be of significant use for genetic research.

Although studies have shown feathers to provide reliable results in the investigation of nDNA, blood is more frequently utilised as it is thought to be a more reliable source. However, Ellegren (1992) extracted nDNA from the inferior umbilicus of flight feathers and found this derivative to be an extremely useful resource for DNA fingerprinting, being easier to collect and store than other materials. In this article, Ellegren (1992) also suggested for the first time the possible use of moulted feathers for genetic analysis.

3.5.1 Non-invasive feather sample collection

Feathers can be obtained from a number of sources, do not require specialised storage and generally provide a good quantity and quality of DNA. Feather sampling is therefore an extremely useful method for those working in the field where equipment may be minimal and samples opportunistic. Obtaining samples from birds in captivity is obviously a simpler process than collecting in the field and presents less opportunity for contamination and misidentification. However, if a reference sample can be obtained for comparative purposes of the species under study, feathers which appear morphologically similar can be collected in the field for genetic identification. A misidentification at the morphological level can therefore be overturned at the genetic level. For example, to expedite the process of species identification of remotely collected samples in an investigation of the Eastern Imperial Eagle (*Aquila heliaca*) by Rudnick et al., (2007) restriction fragment length polymorphisms were used to identify samples.

Determining feather origin is an important consideration when working with moulted samples as identification is the key parameter to non-invasive sampling. If a feather is not seen to moult from a recognisable species and remote collection occurs it is important to establish identification, especially if the feather contains no unique features and may therefore correspond to a number of species present in the area. If no method of identification is established for the

study this may result in the analysis of samples from non-target species. Not only are the results obtained unusable to the investigation but performing full analysis on the incorrect sample is an unnecessary expenditure. By creating a simple method of identification, samples can be processed to identify those indicative to the investigation. Feather samples can initially be compared to a reference sample to identify their origin if morphologically distinctive as well as performing barbule analysis to attempt classification of the sample. If a reference sample is not available to researchers, resources such as The Feather Atlas available on the U.S. Fish and Wildlife Service Forensic Laboratory website can be used to compare unknown feathers (The Feather Atlas, 2010).

3.5.2 Examination of feathers prior to genetic testing

Hogan et al., (2007) developed a simple method of visual analysis to estimate the probability of obtaining viable DNA from a feather sample. 637 moulted feathers were used to observe the effects of feather type and condition, based on the success of amplification of nuclear and mitochondrial DNA markers. The mtDNA amplifications were more successful than nDNA; however this would be expected due to the higher quantity of mtDNA available. Not expected in this study was the result that down feathers were more successful than flight feathers for mtDNA extraction as apparently down feather quality did not have an effect on the amplification. One factor which may have influenced this result is the quality of down feathers being more difficult to establish due to the composition of the feather. Moulted down feathers will degrade more rapidly in the environment as complete degradation may occur in a matter of days compared to months with a semiplume or flight feather. A non-invasively collected down feather may therefore be fresher than other feather types obtained in the field. Booms et al., (2008) demonstrated this effect by investigating feather endurance and found flight feathers to remain the longest in the field. In the study by Hogan et al., (2007) 7 poor quality down feathers were obtained (8% of the total poor quality feathers) demonstrating the poor

persistence of this feather type. The study demonstrated that the quality of the feather collected would have more influence than feather type over the success of DNA extraction, a finding which has been substantiated in subsequent studies (Gebhardt, Brightsmith, Powell & Waits, 2009).

3.5.3 Common feather types in genetic studies

Feathers used for DNA extraction are normally flight, semiplume or down feathers. However, when working with museum samples, flank feathers will usually be provided as those of the tail and wing are used for measurement and will not be removed. In this case, a larger section of the feather shaft must be utilised than when working with feathers from live birds in order to obtain a sufficient quantity of DNA to undergo further analysis.

When undertaking non-invasive sampling, semiplume feathers will most likely be obtained for analysis as they are more frequently moulted and of an appropriate size for performing DNA extractions. The size of the feather obviously relates to the size of the bird and in some cases the proportions of the feather shaft can be identical in the semiplume feathers of one bird and the flight feathers of another. The number of feathers which are required for analysis will therefore depend upon the species of study and the selection of feathers for analysis can be conducted using the visual analysis described by Hogan et al., (2007). In addition, Segelbacher (2002) concluded that if moulted feathers were to be used for analysis then twice the quantity of the required sample size should be obtained to ensure success.

Although down feathers may be of great use to those collecting samples from nesting sites, care must be taken to ensure the nest is not insulated with feathers which did not originate from the species. The nest of a long tailed tit (*Aegithelos caudatus*) is a prime example of this, containing hundreds of feathers collected from the environment. It is therefore not feasible for investigations studying species such as this to adopt nest sampling methodologies. Researchers must also incorporate aspects of animal behaviour into their study to account for species which, for example, are known to steal or

use the nests of other birds and thus potentially confuse sample collection. With respect to the Trinidad Piping guan, only in recent years has the study of nests been possible and it appears the principle materials used are leaves and twigs making the method unfeasible. In addition, nests are normally found within tree canopies and therefore gaining access to this resource would be problematic.
Although Hogan et al., (2007) found feather type to be of less importance overall compared with quality, Bayard De Volo et al., (2008) have looked in detail at feather type alone and found that rectrices (feathers of the tail) provided significantly more DNA overall than remige (feathers of the wing) and smaller feathers, but were equally successful with regard to PCR amplification.

3.5.4 Location of highest DNA yield within a feather

As well as feather type, the region in which DNA is to be extracted must also be considered. Figure 3.1 demonstrates the main feather components commonly discussed in feather sampling publications.

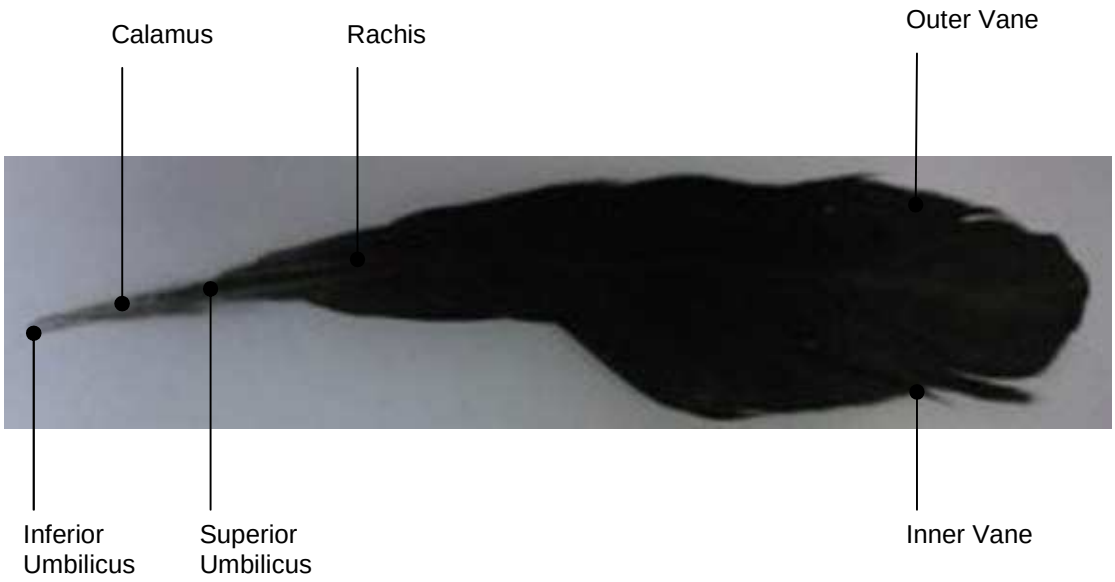


Fig. 3.1: Feather components demonstrated by a wing feather (remex).

Generally, material above the superior umbilicus can be discarded as this section of the feather shaft is small in diameter and contains a limited number of cells feasible for DNA extraction. However, recently the feather rachis and barbs have been found to provide enough mtDNA for species identification. Classification has been performed by amplifying 31-180bp of the D-loop (Rawlence, Wood, Armstrong & Cooper, 2011) and as little as two feather barbs have been used for species identification by amplification of 200-300bp of cyt *b* (Speller, Nicholas, & Yang, 2011). These sections are conventionally used for the gross study of barbules by microscopy in order to help classify a species if feather origin is unknown. The quill can be used for two types of extraction including either the superior or inferior umbilicus, though if performing multiple extractions on a large feather an extraction purely from the calamus is also possible. These two regions each have their own benefits which increase the chance of DNA extraction.

In order to understand the cell type of which DNA is being extracted from, it is important to recognise the late stages of feather growth. When feather growth nears completion the calamus will contain the menschymal pulp within which are situated the axial artery along with numerous other blood vessels and pulp cells, surrounded by the pulp epithelium. The pulp epithelium separates into inner and outer layers, the inner producing pulp caps as the pulp is absorbed during maturation. When completely absorbed a pulp cap forms over the dermal papilla; the feather is now classed as mature and has no blood supply (Yu et al., 2004). During feather development the mesenchymal pulp crosses the calamus wall and a blood clot originating from the axial artery in the region of the superior umbilicus is often observed (figure 1 in Horváth, Martínez-Cruz, Negro, Kalmár & Godoy, 2005). If using the feather to isolate nDNA, the superior umbilicus represents the most viable option for DNA extraction; especially if collected remotely and the time elapsed since moulting can only be speculated.

Plucking a feather results in tissue from the dermis adhering to the inferior umbilicus from which viable DNA can be extracted (Horváth et al., 2005; Bayard De Volo et al., 2008). If conducting non-invasive sampling these skin cells may

still be present but after exposure to environmental conditions contamination and degradation may occur. In addition, latent pulp cells within the feather shaft are also a viable resource for DNA extraction if remaining after absorption.

Commonly, the inferior umbilicus is the preferred sample type due to the chance of remaining adherent skin cells and being the easiest extraction to perform by simply removing the end of the quill. The amount of the quill tip used is purely based on judgement with regard to the size of the feather and its condition. Following removal of the DNA sample, the remaining feather can be kept intact and, if required, further samples taken from the top of the previous cut until reaching the superior umbilicus.

3.5.5 The integrity of non-invasive feather samples

The first use of naturally shed feathers came shortly after the advent of PCR in 1994 when Morin et al., published the results of extracting mtDNA from both plucked and moulted feathers. DNA was extracted from the inferior umbilicus of 1-3 year old feathers and amplified using universal primers designed by Kocher et al., (1989). Morin et al., (1994) found that the size of the feather had little effect on DNA extraction as a larger section of the quill tip could be removed for smaller feathers to match the quantity of material used for bigger feathers. Obviously, a greater section of the quill would begin to incorporate different parts of the feather shaft, up to or including the superior umbilicus.

Segelbacher (2002) conducted an extensive investigation on the different types of feather sampling to demonstrate the integrity of the resource. It was found that large feathers amplified more microsatellite loci than small feathers and that although successful in genotyping, moulted feathers produced less reliable results with approximately half of the samples producing no viable DNA. Though not investigated by Segelbacher, the percentage of moulted feathers producing viable results may have increased if mtDNA markers were also investigated. Although suggesting that not all non-invasive samples may be suitable for genetic analysis, this study demonstrated the feasibility of this method of sample collection. The use of moulted feathers was an important step in obtaining

genetic information from birds as sample collection could occur without capture. In 1996, DNA sexing of birds using moulted feathers was performed by Griffiths, Daan & DijkstraIn; a technique which has since proven widely applicable to many species. A review of non-invasive sampling by Taberlet and Luikart (1999) highlighted the variability in the concentration of DNA extracted from moulted feathers. However, this has since been explained in part by the methods of visual analysis created by Hogan et al., (2007) and the estimation of time the feather has spent in the field prior to collection (Booms et al., 2008).

3.5.6 Increasing the chance of DNA extraction in non-invasive sampling

For non-invasive samples obtained from captive birds, generally the larger and more recently moulted the feather, the greater the chance of successful DNA extraction. Flight feather quills have a larger diameter than all other feather types, providing an adequate quantity of starting material. However, it may take some time to obtain these feathers as they are not moulted as frequently.

If collecting field samples rather than obtaining specimens from captive birds and nDNA is required, small body feathers should be collected. It has been shown that these feathers do not persist in the field as long as flight feathers and therefore the DNA sample will be less likely to have undergone degradation (Booms et al., 2008). The visual analysis method of Hogan et al., (2007) can also be used to identify viable samples reducing the chance of variables such as allelic dropout to provide more reliable results. However, the recovery of mtDNA is less problematic and therefore flight feathers recovered from the field can be used as reliable source. Tail feathers have been shown to produce greater quantities of DNA (Bayard De Volo et al., 2008) and therefore if good quality samples have been collected, these should undergo DNA extraction first. For any given species the comparative size of flight feathers will provide the greatest quantity of material to sustain several DNA extractions. It may therefore be possible to perform all experimental investigations on one feather reducing sample volume and ensuring only one individual from the species is used. This is especially important if a voucher specimen is being studied and limited

samples are available. If flight feathers are not attainable it has been shown that successful mtDNA extraction is possible from the majority of feather types, the most important factor being the condition of the sample (Hogan et al., 2007; Gebhardt et al., 2009).

3.6 DNA extraction, amplification and subsequent electrophoresis

To test the efficiency of the chosen methodology, DNA was extracted from moulted feathers received from the captive specimen of the Trinidad Piping guan which was held at the Emperor Valley Zoo in Trinidad. The method of visual analysis described by Hogan et al., (2007) was used to select two of the six feathers received for DNA extraction. DNA was isolated from the inferior umbilicus of both feathers with Qiagen DNeasy blood and tissue kit using a 5mm sample from a tail feather and a 10mm sample of a semiplume feather. DNA was then amplified using primers and PCR conditions listed in tables 3.4 – 3.6 (primer sequences can be found in Appendices II) using Amersham Biosciences PuReTaq Ready-To-Go 0.2ml PCR beads and a Techne Flexigene thermal cycler. All reactions were set up in a UV cabinet using conditions described in chapter 2. Amplicons were visualised on a 2% agarose gel pre-stained with GelRed™ and run against 10µl of Norgen Biotek Corporation PCRSizer 100bp DNA ladder. 2µl of 6X blue loading dye was added to 10µl of each PCR amplification and inserted into the wells of the agarose gel which was run at a constant 100V. Gels were visualised using a ChemiDoc™ XRS UV transilluminator and images captured using BioRad Molecular Imager®, formatted with Quantity One 1D analysis software.

Results

3.7 Methodology selection for the current investigation

All sampling methodologies and considerations for each approach were examined for use in the current study and a table summarising the advantages and disadvantages of feather sampling was created (see table 3.3). In addition, a flowchart was devised to assist researchers in the selection of a sampling

methodology in avian studies (see figure 3.2). Methods such as the collection of guano and buccal swabs have been intentionally excluded from this figure as these techniques are not necessarily applicable to all species.

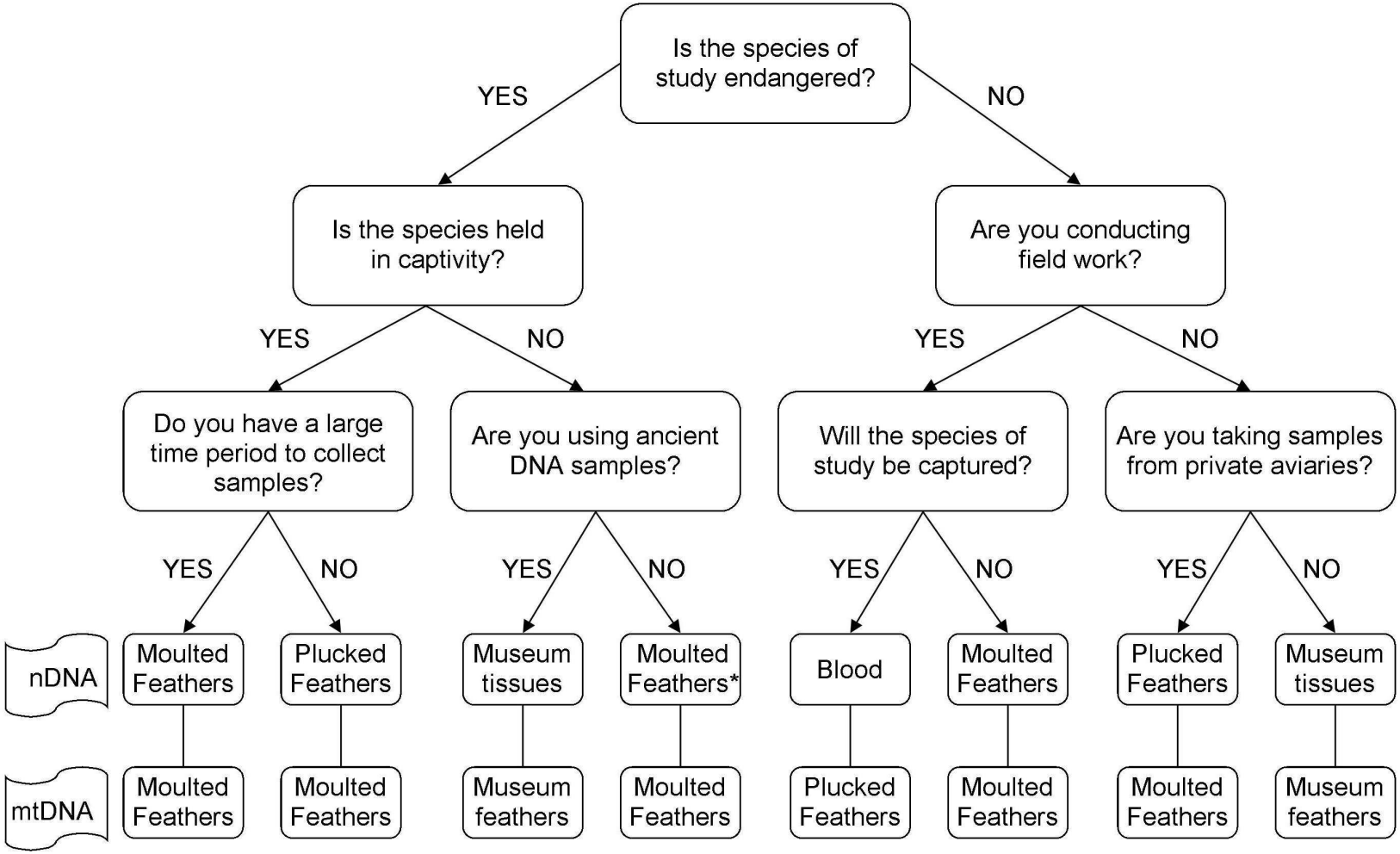
It was found through literature review and use of the devised flowchart that the most viable resource for genetic sampling of the Trinidad Piping guan was non-invasive feather collection.

3.7.1 Results of the chosen sampling methodology

Figures 3.3 - 3.5 with associated tables 3.4 - 3.6 highlight the amplification of mtDNA from moulted Trinidad Piping guan feathers using a mixture of universal, avian and designed primer sets. All amplifications were performed using unpurified DNA extracted from moulted feathers obtained from the captive Trinidad Piping guan. The quantity of DNA obtained in the PCR amplification reactions differs depending on the feather sample on which the extraction was performed and the specificity of the primers to the species.

Table 3.3: Summary of the advantages and disadvantages of feather sampling methods discussed in the text.

	Advantages	Disadvantages
Mature plucked feathers	Greater chance of nDNA recovery. Origin known. Less opportunity for contamination.	Training required to pluck feathers. Causes stress to the bird. Body feathers are often plucked providing a smaller sample.
Moulted feathers	No stress is caused to the bird. Opportunistic collection for endangered species. Origin is known if held in captivity. Easy visual analysis to estimate DNA. Minimal training is required for sample collection. Chance of obtaining flight feathers.	A reference sample is required to ensure origin of feathers collected in the wild. Less viable nDNA. Requires more time for samples to become available.



*Plucked feathers or blood could be collected opportunistically if the species is to be captured via another research project

Fig. 3.2: Flowchart devised in the current study for assisting the selection of a study design for avian genetic investigations.

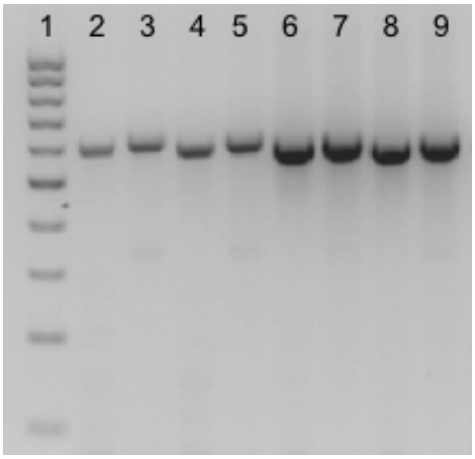


Fig. 3.3: Amplification of DNA extracted from captive Pipile pipile *samples using ND2 primers. Primers used for amplification are described in table 3.3 (See Appendices II for primer sequences). Lanes 2-5 utilised DNA extracted from a semiplume feather and those seen in lanes 6-9 were amplified using DNA extracted from a flight feather. In each case a single band of the expected size is evident demonstrating primer specificity and appropriate PCR conditions. Lanes 2-3 and 6-7 used the first elution from the DNeasy wash step and lanes 4-5 and 8-9 used the second elution.*

Table 3.4: Primers used to amplify regions of the ND2 gene, lane 1 contains a 100bp ladder.

Lane	DNA region	Forward Primer	Reverse Primer	PCR Conditions
2	ND2	L5216	H5766	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
3	ND2	L5758	H6313	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
4	ND2	L5216	H5766	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
5	ND2	L5758	H6313	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
6	ND2	L5216	H5766	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
7	ND2	L5758	H6313	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
8	ND2	L5216	H5766	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
9	ND2	L5758	H6313	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$

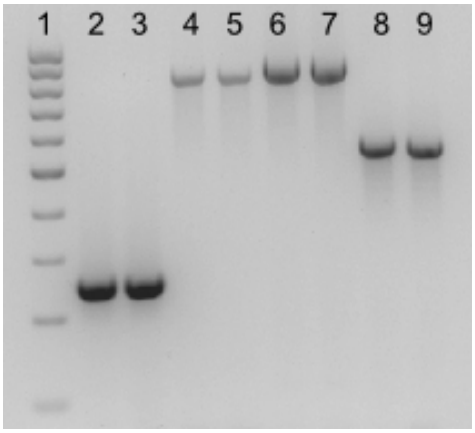


Fig. 3.4: Amplification of DNA extracted from captive Pipile pipile *samples using ND2, COI and WANCY (RNA cluster) primers. Primers used for amplification are described in table 3.4 (See Appendices II for primer sequences). All amplifications were performed using DNA extracted from flight feathers. Lanes 2-3 contain the primers used to amplify the middle of the ND2 gene, lanes 4-5 show the first half of COI, lanes 6-7 the second half of COI and lanes 8-9 the amplification of the WANCY tRNA cluster found between ND2 and COI, joining the gene regions. Again single bands are evident for each reaction demonstrating clean amplification.*

Table 3.5: Primers used to amplify regions of the ND2, COI and WANCY genes, lane 1 contains a 100bp ladder.

Lane	DNA region	Forward Primer	Reverse Primer	PCR Conditions
2	ND2	Lc5568	Hc5608	$94^{5}(94^{1}55^{1}72^{2})$x40 72^{30}
3	ND2	Lc5568	Hc5608	$94^{5}(94^{1}55^{1}72^{2})$x40 72^{30}
4	COI	LTyr	H763	$94^{5}(94^{.40}50^{.40}72^{1})$x40 72^{7}
5	COI	LTyr	H763	$94^{5}(94^{.40}50^{.40}72^{1})$x40 72^{7}
6	COI	L674	H8205	$94^{5}(94^{.40}50^{.40}72^{1})$x40 72^{7}
7	COI	L674	H8205	$94^{5}(94^{.40}50^{.40}72^{1})$x40 72^{7}
8	WANCY	L6182	H6735	$94^{5}(94^{1}60^{1}72^{1})$x40 72^{5}
9	WANCY	L6182	H6735	$94^{5}(94^{1}60^{1}72^{1})$x40 72^{5}

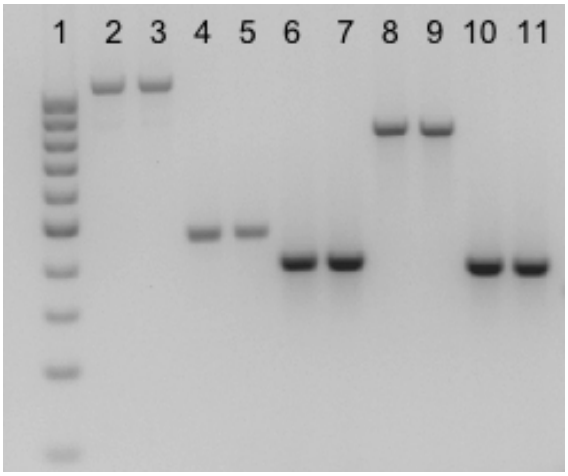


Fig. 3.5: Amplification of DNA extracted from captive Pipile pipile *samples using ND6, D-loop and cyt* b *primers. Primers used for amplification are described in table 3.5 (See Appendices II for primer sequences). All amplifications were performed using DNA extracted from flight feathers. Lanes 2-3 contains the amplification of ND6, lanes 4-7 show partial amplifications of the D-loop, lanes 8-9 contain partial amplification of cyt* b *and lanes 10-11 contain another D-loop amplification designed to incorporate the region of the bird similarity box. Clean amplification and primer specificity is demonstrated by a single strong band.*

Table 3.6: Primers used to amplify regions of the ND6 and Cyt b genes and non-coding D-loop, lane 1 contains a 100bp ladder.

Lane	DNA region	Forward Primer	Reverse Primer	PCR Conditions
2	ND6	Lcytb	Hdloop	$94^{5}(94^{.30}60^{.30}72^{1})x30\ 72^{5}$
3	ND6	Lcytb	Hdloop	$94^{5}(94^{.30}60^{.30}72^{1})x30\ 72^{5}$
4	D-loop	774L REV	H1251 REVI	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
5	D-loop	774L REV	H1251 REVI	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
6	D-loop	L436 REVI	H774	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
7	D-loop	L436 REVI	H774	$94^{5}(94^{1}55^{1}72^{2})x40\ 72^{30}$
8	Cyt *b*	L15311	H16065	$94^{5}(94^{1}55^{1}72^{1})x40\ 72^{5}$
9	Cyt *b*	L15311	H16065	$94^{5}(94^{1}55^{1}72^{1})x40\ 72^{5}$
10	D-loop	L465	H839	$94^{5}(94^{.30}60^{.30}72^{1})x30\ 72^{5}$
11	D-loop	L465	H839	$94^{5}(94^{.30}60^{.30}72^{1})x30\ 72^{5}$

3.8 Discussion

The amplification of a range of mitochondrial genome regions of varying sizes demonstrates that non-invasive feather sampling is an adequate method for the current study. Results concur with Bayard De Volo et al., 2008 as a higher quantity of DNA is seen to amplify with ND2 primers when using DNA extracted from a tail feather rather than a semiplume feather, seen in figure 3.3. The final elution with buffer AE when using the DNeasy extraction kit was conducted in two wash steps which were compared under identical conditions and produced a similar quantity of DNA amplicon (figure 3.3). As the flight feathers produced superior amplifications, these DNA extractions were continued for use in further PCR reactions. Primers were designed to amplify an overlapping section of the previous ND2 reactions and because of their specificity to the species can be seen to produce a strong amplification (figure 3.4). Amplification was then performed in an additional protein coding gene (COI) and within a tRNA cluster, all providing sufficient quantity for subsequent sequencing. Finally, to examine the gene used for species identification (cyt *b*) and notoriously difficult regions to amplify due to high levels of variation (ND6 and D-loop) PCR reactions were performed to examine the size of the fragment which could be amplified using non-invasive samples (figure 3.5). Fragments from 477bp to over 1000bp were successfully amplified, demonstrating that good quality and quantity DNA had been recovered from the moulted feather samples. The feathers on which these tests were performed were taken from the enclosure of a captive bird and were therefore quickly recovered, reducing the time for contamination and degradation, and stored at room temperature until export. The results give promise to samples collected in the field as many reactions produced amplicons of over 78ng (the highest value available for comparison on the PCRSizer) of which only 30ng is required for sequencing. If feathers received are sub-optimal and some degradation has occurred, a reduced amplification may therefore still be sufficient for further analysis.

In addition, moulted feather samples were requested from closely-related and out-group species throughout the course of the study so as to avoid stress to the

birds and remove the necessity of trained personnel for sample collection. This method is also acceptable for use by conservation parks as no disruption is caused to captive animals which may be part of breeding programmes. When museum feather samples were required, feather sampling was adopted to cause minimal destruction to the exhibit whilst still obtaining a viable sample. Samples were to be removed from the inferior umbilicus to try and avoid possible contamination and keep the feather in good condition should it need to be returned to the museum. The slower degradation time and higher copy number of mtDNA, coupled with the protection provided by the keratin sheath of the feather quill assists the preservation of genetic material enabling large gene fragments to be amplified. The success of larger amplifications will decrease with remotely collected field samples and museum samples, however the majority of amplifications in the current study are <1000bp making amplification more viable.

Remotely collected Trinidad Piping guan samples required a method of genetic identification to verify origin which was possible through the use of samples obtained from the captive specimen. Subsequent sequence data enabled the creation of field markers which is discussed further in chapter 5.

Due to feather samples having a longer timeframe for collection than materials such as faeces, viable mtDNA can be obtained from remotely collected samples which may have remained in the field for a number of weeks. DNA is partially protected from environmental conditions due to the structure of the feather and is durable enough to undergo a wash step before DNA extraction if required. This wash step may be performed if a feather is received in a poor condition to remove possible contamination and PCR inhibitors. Several primers used in the current study were designed from universal sequencing to allow the amplification of full gene regions in overlapping segments. Many published primer sets were unsuccessful and therefore primer combinations from multiple publications were used in order to achieve sequencing (see Appendices II).

3.9 Conclusion

A review of sampling techniques was undertaken and after considerable research into the methods of biological sampling from Aves, non-invasive feather sampling was shown to be the most appropriate method for the current investigation. This method provided viable DNA from moulted feathers therefore minimising disruption to the remaining population. The moulted feather sampling methodology was shown to be successful as judged by the amplification of mtDNA fragments ranging from ~200bp to >1000bp. All amplifications were seen to produce quantities of DNA suitable for purification and sequencing with no double-banding which would indicate a lack of primer specificity. From the examination of the current literature, a flowchart was created to expedite the decision of sampling methodology for future researchers studying avian species. Due to the elusiveness of the Trinidad Piping guan, samples could be collected opportunistically from known habitat when visited by researchers as no personnel in Trinidad are directly linked to the current investigation for sample collection. Sampling was therefore reliant on the remote collection of materials by researchers studying the health and habitat of the species who kindly collected and exported materials which could be obtained opportunistically. By adopting non-invasive methods, researchers were not required to undergo specialised training or carry additional equipment. Where multiple feathers were obtained, the method of visual analysis created by Hogan et al., (2007) was conducted in order to select the feather with the greatest chance of providing sufficient DNA. When available, tail feathers were chosen for DNA extraction as these have been shown to produce the highest quantity of viable DNA.

Chapter 4

Comparative sequence analysis and phylogenetic reassessment of the Trinidad Piping guan

Introduction

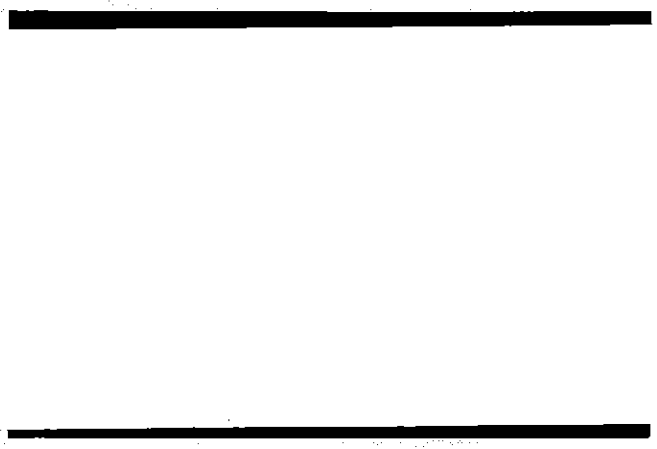
4.1 Phylogenetic status of the Piping guans

The previous phylogenetic assessment of Piping guans devised by Grau Pereira, Silveira, Höfling & Wajntal (2005) (see chapter 1 figure 1.2) is the most comprehensive study of the genus based on genetic data. However, *Pipile pipile* was represented by biological samples obtained from a single specimen held at a private aviary in Mexico. The current study provided opportunity to revise the phylogeny using sequence data from a true island bird. Data could therefore also be used to investigate the unexpected divergence time obtained by Grau et al., (2005) and the disputed classification of *Aburria/Pipile* of Piping guans. The proposal to merge *Pipile* within *Aburria*, as stated previously, was rejected by SACC (Remsen et al., 2007) and is currently recognised by IUCN, IOC and Bird Life as *Pipile*. Despite this rejection, the *Aburria* classification is used by some researchers and the International Taxonomic Information System currently lists taxa under both *Pipile* and *Aburria* but states that *Pipile* is invalid (retrieved November, 2011 from http://www.itis.gov/).

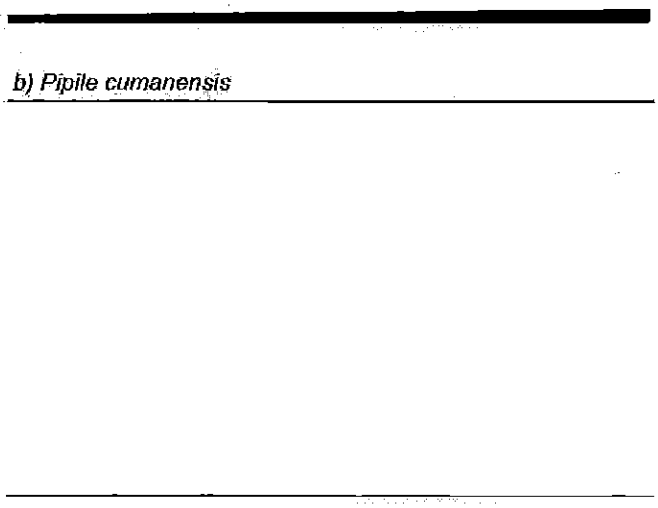
Pipile cumanensis grayi was not incorporated in the original phylogenetic study conducted by Grau et al., (2005) as no sub-species were included. Although it would be expected that *Pipile cumanensis grayi* would branch adjacent to *Pipile cumanensis*, the genetic relatedness was previously unstudied. Due to the morphological characteristics of *Pipile cumanensis grayi* being similar to that of *Pipile pipile* (see figure 4.1a-c) it may be possible that a sub-species relationship exists. However, many factors such as genetic mutation and homology are a possibility in this instance and relatedness cannot be assumed without genetic study. Although *Pipile cumanensis grayi* has been included in later relationship studies (Frank-Hoeflich et al., 2007) four of the five trees drawn did not include

Pipile pipile and were based purely on morphological characteristics. The single tree which represented both *Pipile cumanensis grayi* and *Pipile pipile* was drawn from the analysis of osteological characteristics. Prior to the current study, the relatedness of *Pipile cumanensis grayi* to *Pipile pipile* had not been assessed from genetic data and remained unclear. As such, *Pipile cumanensis grayi* was considered an important comparator species in the present investigation.

a) *Pipile pipile*



b) *Pipile cumanensis*



c) *Pipile cumanensis grayi*

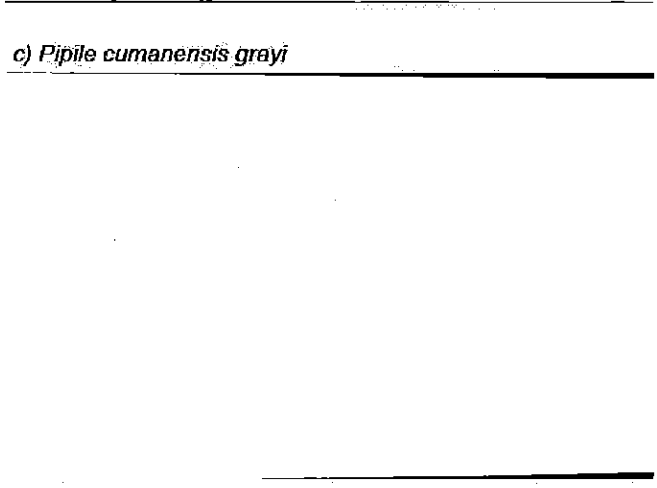


Fig. 4.1a-c: Morphological comparison of Pipile pipile, Pipile cumanensis *and* Pipile cumanensis grayi *clearly indicating the morphological similarities between* Pipile pipile *and* Pipile cumanensis grayi. Photographs provided by a) Margaret Cooper b) Patrícia Lopes c) Louise Peat

4.2 The importance of voucher specimens in genetic investigations

It has frequently been stated that the use of voucher specimens in genetic study is imperative to the validity of results, especially when undertaking studies in systematics when misrepresentation could alter the understanding of evolutionary history (Leeton, Christidis & Westerman, 1993; National Science Foundation, 2000; Bates, Bowle, Willard, Voelker & Kahindo, 2004). The National Oceanic and Atmospheric Association define a voucher species as *'Any specimen that serves as a basis of study and is retained as a reference. It should be in a publicly accessible scientific reference collection'* (NOAA, 2010).

Without this voucher representation, studies cannot be replicated or advanced by future researchers. In addition, it may be impossible for researchers who acquire samples from a non-voucher collection to confirm identification of the material received (Funk, Hoch, Prather & Wagner, 2005). Misidentification by both researchers and museums has been highlighted in previous studies where misclassified genetic material has been deposited on the National Centre for Biotechnology Information (NCBI) genetic database, known as 'GenBank' (Payne & Sorenson, 2002; Linacre & Tobe, 2011). Although some researchers believe those undertaking genetic studies using non-voucher specimens should also obtain a voucher representative, dispensation is given for those studying marked or endangered populations (Bates et al., 2004).

Due to the nature of the present study, no voucher specimens were obtained for the Trinidad Piping guan. However, for *Pipile cumanensis grayi*, representative voucher feather samples were acquired from the Museu de Zoologia da Universidade de São Paulo for the current study (Luís Silveira, personal communication, April 16, 2008).

4.2.1 Reference Sampling

Because no voucher specimen was obtained for *Pipile pipile*, a reference sample of the species was acquired. Genetic analysis using a reference sample has previously been criticised as classification is conducted either by the researcher or by a collector/handler. With respect to remotely collected samples,

the decision regarding species origin is conducted in the field where no direct comparator is available. Although species origin can be confirmed by genetic testing (as mentioned in chapter 3) if no comparator is available for study this analysis cannot be conducted. Researchers are therefore reliant upon the classification awarded by collectors or possibly their own observations. Species identification is therefore often defined by the analysis of factors such as morphology and geographical locality.

An argument against the reproducibility of reference-based studies has been presented by Peterson et al., (2007) as the samples on which analyses are based may not be available to researcher's in future genetic investigations. Though criticising the use of non-voucher specimens, Peterson et al., (2007) did comment that this type of sampling may be necessary when working with endangered species if no voucher is available (such as the present study). In a response to the article by Peterson et al., (2007) it was argued by Olsson, Sundberg, Alström, Gelang & Ericson (2008) that '*Although feathers and blood are evidently not as good as voucher specimens, we do not agree that such material is useless. In our opinion, photographs and/or sound recordings are a reasonable substitute for voucher specimens, when vouchers cannot be collected'.* The authors made four recommendations when collecting non-voucher specimens to assist species verification and provide evidence to support the decision made in the field. This involved the collection of photographs, biometrics, sound recordings and locality description. Though collection of this support material may not be possible, these points should be considered by researchers undertaking reference sampling.

In the present study, the bird which was chosen to represent the Trinidad Piping guan originated from the only known captive individual of the species (a bird which has never left the island) previously held at the Emperor Valley Zoo in Trinidad. Recommendations for non-voucher collection are satisfied as the reference specimen underwent studies by zoologists and veterinarians regarding issues such as external parasites, blood picture and handling (Pawi Study Group, n.d.). Photographs of this reference specimen within its enclosure

and being examined for health studies can be seen in figures 4.2 and 4.3. It was therefore suitable for this captive bird to be used as a reference, rather than voucher, specimen.

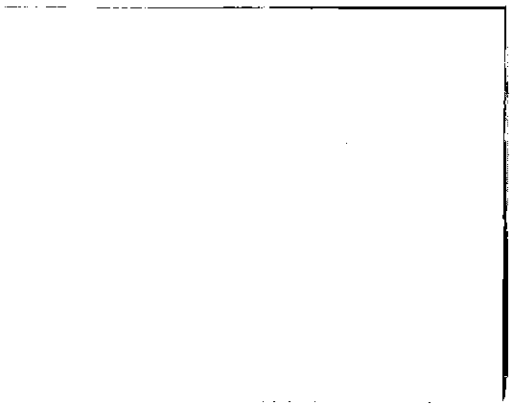


Fig 4.2: Captive Trinidad Piping guan held at the Emperor Valley Zoo in Trinidad.
Photograph provided by Margaret Cooper

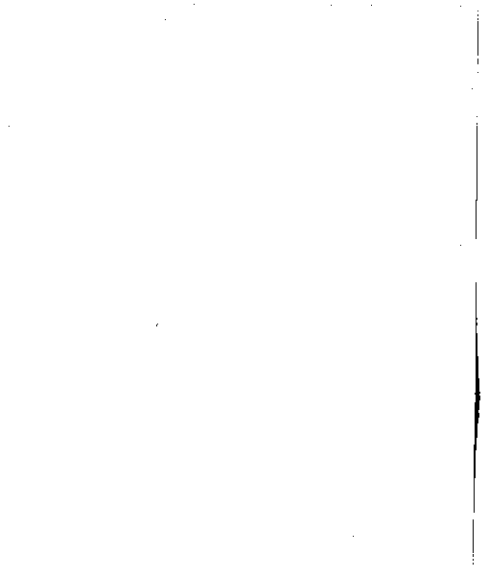


Fig. 4.3: Captive Trinidad Piping guan being examined for health studies.
Photograph provided by Kerrie T. Naranjit

4.3 Single Nucleotide Polymorphisms to distinguish species

Single Nucleotide Polymorphisms (SNPs) identify variation seen within specific regions of DNA and are found within all genomic data (Garvin, Saitoh & Gharrett, 2010). As opposed to anonymous variation seen when using techniques such as Amplified Fragment Length Polymorphisms (AFLPs) the sequence variation can be identified and used as a method of species identification (Belfiore, Hoffman, Baker & DeWoody, 2003; Köhnemann & Pfeiffer, 2010) as well as providing data for population studies, classification and trait mapping (Garvin et al., 2010). Although SNP data can be used exclusively (Webb & Allard, 2009), they are often used in conjunction with other methods as the subsequent analysis is less time-consuming. These methods include Restriction Fragment Length Polymorphisms (RFLPs) (Wolf, Rentsch & Hübner, 1999), primer design (Cassel-Lundhagen, Öberg, Högfeldt & Ekbom, 2009) and Forensically Informative Nucleotide Sequencing (FINS) (Bartlett & Davidson, 1992) which can be used in combination (Wen, et al., 2010) and is discussed further in chapter 5.

Analysis of SNP variation between species can help define cryptic species but an unfair weighting must not be placed on a single location without extensive study of the gene region to class base variation as a species defining SNP. Calculating overall percentage similarity obtained through FINS of closely related species can sometimes cloud variation if the differences observed are within a few percent. When genetic variation is low it is beneficial to analyse sequence data and look for inter-species SNPs to assist classification. With respect to the current study, the analysis of SNPs was used to observe variation between captive Trinidad Piping guan held in Mexico and Trinidad (intra-species variation) and closely related species (inter-species variation). These inter-specific SNPs have been found to be 3-6 times greater in some avian species than in humans making them useful identification markers (Primmer, Borge, Lindell & Saetre, 2002).

4.3.1 The use of mitochondrial DNA in phylogenetics

Originally, morphological data were used to create phylogenies but the use of molecular data for analysis became more frequent as superior laboratory techniques were established (Brown, 2002). Although nDNA data is used within phylogenetics, analysis is commonly based on mitochondrial sequence data or a combination of the two. MtDNA is of great value when resolving phylogenetic trees (especially of recently diverged species) due to the high rate of nucleotide evolution and lack of recombination. Though missense mutation is constrained, the silent (or neutral) mutations which are often observed can be used to distinguish periods of shared ancestry, termed synapomorphies (Mindell, 1997). These substitutions must occur at a rate suitable for separating species without 'multiple hits' occurring (Mindell, 1997). This is observed when DNA has become saturated and recurrent substitutions can obscure the phylogenetic signal (Zink & Barrowclough, 2008). Calculations may therefore become inaccurate as mathematical models no longer fit the data (Dopazo & Brustin, 2009).

Provided sufficient variation is seen between mtDNA haplotypes, a high level of congruence (0.95) has been observed between gene trees (reconstructed from comparing orthologous genes) and species trees (created using morphological characteristics) compared with 0.62 when using a single nuclear gene tree (Moore, 1995). Incongruence between gene and species trees could however occur through hybridization (Moore, 1995) as maternal inheritance could lead to a misrepresentation of the species genetic sequence or its characteristics. Congruent trees are not necessarily identical as the internal nodes of a gene tree are defined by mutation creating a different DNA sequence whereas those in the species tree are defined by speciation events such as the isolation of populations which can no longer interbreed (Brown, 2002). These events may not occur simultaneously therefore for a time, a divided population may contain all haplotypes seen in the original population. Only after events such as genetic drift where alleles are lost does the genetic sequence of the two populations begin to differ. It is assumed that trees based on molecular data will provide a

more accurate representation of the tree and its evolutionary history as the data on which it is based are less ambiguous (Brown, 2002).

4.3.2 ND2 gene

As ND2 has been demonstrated to show a high level of inter-species variation (Sorenson, Ast, Dimcheff, Yuri & Mindell, 1999) and has been used in previous genetic investigations of *Pipile*, the ND2 gene was selected for a comparative and phylogenetic analysis of the genetic variation of Piping guans. As a result of the previous phylogenetic assessments conducted by Pereira & Baker (2004) and Grau et al., (2005), sequence data from ND2 can be retrieved from GenBank for species of Cracid for which genetic samples were unobtainable.

The ND2 gene has a higher rate of first and second base codon mutations making it more suitable for identification between closely related species (Sorenson, 2003). In this respect, ND2 is the third most variable gene in the mitochondrial genome with only ATPase8 and ND6 having a higher level of mutation (Sorenson, 2003). ATPase8 is too short to be phylogenetically informative (162bp) and ND6 is notoriously difficult to amplify due to its placement adjacent to the control region (Sorenson, 2003). Transitions do not appear to reach a plateau in ND2 as is seen in other genes, helping to alleviate the problem of saturation. In spite of the high mutation rate, previous research has shown that the signal created does not negatively affect the phylogenetic tree produced from this data when compared to other genes. Johnson & Lanyon (1999) investigated the effects of ND2 sequence data on phylogenetic analysis and found that in each calculation, ND2 produced the same output as sequence obtained from Cyt *b*. ND2 is therefore the most viable option to observe genetic distinction between *Pipile pipile* and its closest relatives.

4.3.3 Cytochrome **b** *gene*

The cyt *b* gene was chosen for further analysis as it is the most frequently used data in phylogenetics (Nei & Kumar, 2000) and is commonly used in addition to other mitochondrial sequence information to increase the amount of data on

which phylogenies are based. Using more than one gene allows the inclusion of sequence data which is under differing rates of mutation creating a more robust analysis. With respect to the current study, cyt *b* data from previous studies of Piping guans is also available on GenBank allowing direct comparisons of sequence data. The rate of transversion is higher than that seen in other genes, which is an important factor when calculating the model of evolution to use in phylogenetic analysis (Johnson & Lanyon, 1999). A higher transversion rate provides more statistical weighting which can help separate species.

4.4 Phylogenetic models

DNA evolves at a consistent rate, therefore mathematical models can be used to calculate this rate of evolution and compare it to that of other species. This comparison of species genetic data allows the construction of evolutionary trees by statistical analysis using the selected models of evolution and correction factors (Nei & Kumar, 2000). There are three core methods available for conducting phylogenetic reconstructions (distance, maximum likelihood and maximum parsimony) and each of these methods contains numerous models of nucleotide substitution for calculating divergence. Each model considers the nucleotide frequencies and parameters for transition or transversion, assigning a different rate of evolution for each depending on the characteristics of the data being studied (Dopazo & Brustin, 2009). If the rates of transition and transversion are not accounted for when constructing a phylogeny this can affect the accuracy of branching pattern and bootstrap support. These models can also be corrected by gamma distances (Γ) which assume a different rate of evolution of each nucleotide site or invariable sites (I) to include nucleotides which are the same for all taxa (Posada, 2008).

To estimate how robust the tree derived from the data is, methods of re-sampling such as bootstrapping can be performed to demonstrate the confidence level of each node of the tree. In bootstrapping, alternate trees are calculated by re-shuffling the data columns (in which replicates may occur) but the total number of columns will not change (Linacre, 2009). This process is

repeated up to 1000 times (the higher the value, the more robust the analysis) and the consensus tree is presented. Therefore, if a value of 90 is observed, it can be inferred that 100 of the 1000 the replicates were variant. A value of 90 or higher gives confidence to the branch and is considered to be the correct grouping. However, some researchers only accept trees with bootstrap levels above that of normal distribution (95%) (Dopazo & Brustin, 2009) due to issues such as ascertainment bias. The current study will adopt a bootstrap value of 90 or higher for strong nodal support.

4.4.1 Choosing a model of nucleotide substitution

It is important to choose the most suitable model of nucleotide substitution and correction method as a model which is too simplistic for the data will underestimate the number of substitutions, affecting the tree topology (Bruno & Halpern, 1999; Posada & Crandall, 2001). The model of nucleotide substitution (also known as the model of evolution) is established by hierarchical likelihood ratio tests using parsimonious principles, calculated with software such as jModelTest (Posada, 2008; Guindon & Gascuel, 2003). Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) analysis are performed to identify the evolutionary model which best fits the data using the minimum number of free parameters (Shapiro, Rambaut & Drummond, 2006). Increasing the parameters of a model does not necessarily have a significant effect on results and can in some cases reduce the accuracy of the tree if too many parameters are included. The most complicated model of evolution is therefore not necessarily the most superior for the data. If parameters can be excluded, this reduces the noise of the analysis and only includes free parameters which have a significant effect on the results (Foster, 2010). No model of evolution will ever be 'true' to that which generated the data, but can approximate reality to a best fit scenario (Posada & Buckley, 2004). In the current study, the model of evolution was selected by performing AIC and BIC calculations with jModelTest to find that which suited the data of the current study.

Methods

4.5 DNA extraction, amplification & sequencing of feather samples

DNA was extracted from the inferior umbilicus of feather samples using Qiagen DNeasy blood and tissue kit following a user developed protocol. 5mm of *Pipile pipile* feather quill and 10mm of the voucher *Pipile cumanensis grayi* feather quill was used for DNA extraction. ND2 and Cyt *b* genes were amplified with the primers described in table 4.1 using the cycling parameters described in chapter 2 for *Pipile pipile* and *Pipile cumanensis grayi*. Double stranded DNA sequencing was performed by MWG Eurofins using the amplification primers and data was received in FASTA format.

Table 4.1: Primers for the amplification of ND2 and Cyt *b* genes.

Gene	Primer	Sequence 5' – 3'	Derived from
ND2	L5216	GGCCCATACCCCGRAAATG	Sorenson (2003)
ND2	H5766	RGAKGAGAARGCYAGGATYTTKCG	Sorenson (2003)
ND2	L5758	GGNGGNTGAATRGGNYTNAAYCARAC	Sorenson (2003)
ND2	H6313	ACTCTTRTTTAAGGCTTTGAAGGC	Sorenson (2003)
ND2	L5568	AGGATCGCACCTAACCACTGCC	Current study
ND2	H5608	GTAGGCTGAGTTTGGGGCTA	Current study
Cyt *b*	ND5	TAGCTAGGATCTTTCGCCCT	Thomassen (1989)
Cyt *b*	CytbD	TGTAGGTTGCGGATTAGCCAGC	Current study
Cyt *b*	L14990	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	Kornegay (1993)
Cyt *b*	H15696	AATAGGAAGTATCATTCGGGTTTGATG	Kornegay (1993)
Cyt *b*	H15670	GGGTTACTAGTGGGTTTGC	Kimball (1999)
Cyt *b*	L15311	CTCCCATGAGGCCAAATATC	Kimball (1999)
Cyt *b*	H16065	TTCAGTTTTTGGTTTACAAGA	Kimball (1999)

4.5.1 Alignments and pairwise comparisons

The data obtained in the present study from the island specimen of *Pipile pipile* and the voucher specimen of *Pipile cumanensis grayi* (from this point referred to as 'reference *Pipile pipile'* and 'voucher *Pipile cumanensis grayi*') were compared to data available on GenBank from the previous phylogenetic studies (termed 'GenBank *Pipile pipile'* and 'GenBank *Pipile cumanensis grayi*'). Sequence data was aligned using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997) to identify SNPs for species comparison and subsequently used to calculate pairwise distances and perform phylogenetic analyses with jModelTest and MEGA4 (Posada, 2008; Tamura, Dudley, Nei & Kumar, 2007). The GenBank accession numbers of the data used in sequence SNP comparison and for phylogenetic analysis can be seen in table 4.2 and 4.3 respectively.

Table 4.2: GenBank accession numbers of sequence data used in collaboration with sequence data obtained in the present study for *Pipile pipile* and *Pipile cumanensis grayi* for sequence analysis by alignment and pairwise comparisons.

Species	Gene	Accession number
Pipile pipile	ND2	AY367094
Pipile pipile	ND2	AY367100
Pipile cumanensis	ND2	AY367093
Pipile cumanensis	ND2	AY367099
Pipile cujubi	ND2	AY367092
Pipile cujubi	ND2	AY367098
Pipile jacutinga	ND2	AY140744
Pipile pipile	Cyt *b*	AY367106
Pipile cumanensis	Cyt *b*	AY367105
Pipile cumanensis grayi	Cyt b	AY659797
Pipile cujubi	Cyt *b*	AY367104
Pipile jacutinga	Cyt *b*	AF165476

Table 4.3: GenBank accession numbers used in collaboration with sequence data obtained in the present study for *Pipile pipile* and *Pipile cumanensis grayi* for phylogenetic analysis.

Species	Gene	Accession number
Pipile cumanensis	ND2	AY367093
Pipile cumanensis	ND2	AY367099
Pipile cujubi	ND2	AY367092
Pipile cujubi	ND2	AY367098
Pipile jacutinga	ND2	AY140744
Crax rubra	ND2	AY141935
Pipile cumanensis	Cyt *b*	AY367105
Pipile cumanensis	Cyt *b*	AY659798
Pipile cujubi	Cyt *b*	AY367104
Pipile cujubi	Cyt *b*	AY659799
Pipile jacutinga	Cyt *b*	AF165476
Crax rubra	Cyt b	AY956378

4.5.2 Selecting a model of nucleotide substitution for the current study

In the current study, all data is orthologous (therefore genes do not duplicate during evolution) and is restricted in the number of multiple hits due to the relatively recent evolutionary time between the species, therefore DNA data is not saturated. The Tamura Nei model with invariable sites (TrN+I) was found to be the most appropriate model for the current data set. Although BIC penalises free parameters more strongly than AIC, both criteria recommended the same model. The TrN model assumes three rates of substitution, with all transversions having the same rate and each transition a different rate (shown graphically in figure 4.4) and has 6 free parameters. Likelihood Ratio Tests (LRT) were also performed to further confirm the choice of model, P and LRT values of the compared tests (TrN Vs TrN+I and TrN+I Vs TrN+I+G) also showed TrN+I to be the most significant fit for the data.

Fig. 4.4: TrN rate parameters are defined as a =c =d =f, b, e *modified and translated from the thesis of Sérgio Pereira (2000).*

4.5.3 Construction of phylogenetic trees

The phylogenies were created in MEGA4 (Tamura et al., 2007) in two instances using the neighbour-joining method with an interior branch test of 1000 bootstrap replicates with a single tree drawn using maximum parsimonious principles and the nearest neighbour interchange method with 10 replicates. The analysis was performed using a concatenated sequence of ND2 and cyt *b* data as this approach has been shown to produce more accurate trees (Gadagkar, Rosenberg & Kumar, 2005). Evolutionary distances were computed using the Tamura-Nei method with invariable sites including 1st, 2nd and 3rd base codon positions with a total of 1884 positions in the final data set. The gene tree created by this calculation was therefore used to infer a species tree of Piping guans (Nei & Kumar, 2000).

Results

4.6 Alignment of ND2 sequence data

For sequence comparison, GenBank data for *Pipile pipile* (AY367094 and AY367100) were aligned with the reference *Pipile pipile* data (Pp_C.F.2008/ND2). Because the two ND2 data sets available on GenBank for *Pipile pipile* do not overlap, these were aligned separately. In these comparisons, 5 point mutations were identified in the AY367100 sequence alignment (441bp) and 7 point mutations in the AY367094 alignment (454bp). See table 4.4 for the alignment base position of point mutations. As multiple SNPs were identified in this intra species comparison, the sequencing data from the current study was re-analysed to eliminate the possibility of errors and cross contamination in the laboratory. All sequencing electropherograms were examined at the regions of point mutation but no mixed or poor signals were present and all sites produced a phred score of ≥30 (confidence of ≥99.9%).

In an attempt to account for the high level of variation observed between the *Pipile pipile* data sets (variation would be expected to be 1 or 2 point mutations within this length of sequence data) the reference *Pipile pipile* sequencing was subsequently aligned with sequence obtained from the voucher specimen of *Pipile cumanensis grayi* (Ppg_V.F.2009/ND2). From this alignment, 14 point mutations were identified of which 12 were observed in the alignments of reference *Pipile pipile* and GenBank *Pipile pipile* data. The 2 remaining point mutations were seen in the region for which no sequence information for the GenBank *Pipile pipile* is available. Subsequently, when the GenBank *Pipile pipile* data was aligned with the voucher *Pipile cumanensis grayi* data no point mutations were observed in either alignment.

Table 4.4: Point mutations in *Pipile pipile* and *Pipile cumanensis grayi* ND2 sequence alignment.

Sequence Comparison	SNPs	Base position of mutation in sequencing alignment													
Reference *Pipile pipile* Vs *Pipile pipile* AY367100	5	83	274	334	358	447	/	/	/	/	/	/	/	/	/
Reference *Pipile pipile* Vs *Pipile pipile* AY367094	7	/	/	/	/	/	/	/	616	679	700	734	739	844	937
Reference *Pipile pipile* Vs *voucher Pipile cumanensis grayi*	14	83	274	334	358	447	556	574	616	679	700	734	739	844	937
Voucher *Pipile cumanensis grayi* Vs *Pipile pipile* AY367100	0	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Voucher *Pipile cumanensis grayi* Vs *Pipile pipile* AY367094	0	/	/	/	/	/	/	/	/	/	/	/	/	/	/

Summary of sequencing alignments with the base position at which point mutations occurred in the ND2 sequencing alignments. It can be seen that the point mutations in the comparison of the reference *Pipile pipile* sample to GenBank *Pipile pipile* data occur at the same positions as within the comparison of the reference *Pipile pipile* sample with the voucher specimen of *Pipile cumanensis grayi*.

4.6.1 Implications of the ND2 alignment

The 100% similarity match between GenBank *Pipile pipile* and voucher *Pipile cumanensis grayi* over such a large region of sequencing provides evidence that the data currently available on GenBank for *Pipile pipile* may have been incorrectly classified. In this respect, the subsequent match of sequence data with *Pipile cumanensis grayi* rather than *Pipile pipile* would suggest that the data previously classified as *Pipile pipile* in publications and on GenBank originates from *Pipile cumanensis grayi*. All of the genetic data available on GenBank for *Pipile pipile* were obtained from the same specimen housed at Granja 'La Siberia' in Mexico, therefore all sequences deposited on GenBank are under speculation. As this data has been used in previous phylogenetic investigations of Piping guans (Pereira, Grau & Wajntal, 2004; Grau et al. 2005) the results from the present study suggest that the current placement of *Pipile pipile* in the Piping guan phylogeny may be questionable. In this respect, as the previous studies did not include *Pipile cumanensis grayi*, any sequence similarity may not have been immediately apparent. In addition, although *Pipile cumanensis grayi* was included in the later study by Frank-Hoeflich et al., (2007) genetic data of *Pipile pipile* was excluded from this analysis and the similarity between sequencing appears to have gone undetected.

In order to verify the *Pipile cumanensis grayi* sequence data deposited in GenBank, a nucleotide sequence comparison was undertaken. However, as only cyt *b* data is available on GenBank for *Pipile cumanensis grayi*, alignments were performed with genetic data for this gene.

4.6.2 Alignment of cyt b *sequence data*

Variation was expected in the cyt *b* alignment of the reference *Pipile pipile* (Pp.C.F.2008/Cytb) and GenBank *Pipile pipile* sequence data as the GenBank data was derived from the same biological samples as that which generated the ND2 data. When the sequences were aligned, 9 point mutations were observed.

Aligning the voucher specimen of *Pipile cumanensis grayi* (Ppg_V.F.2009/Cytb) with the reference *Pipile pipile* data produced 17 point mutations, 9 of which

were observed in the previous sequence alignment of reference *Pipile pipile* and GenBank *Pipile pipile*. Also in common with the alignments of the ND2 gene, comparison of the voucher *Pipile cumanensis grayi* with the data currently available on GenBank for *Pipile pipile* produced a 100% match (see table 4.5 for the base positions of the point mutations in the alignment). This further supports the hypothesis that the GenBank data for *Pipile pipile* has been derived from an incorrectly classified species.

Subsequently, in order to verify the data currently available on GenBank for *Pipile cumanensis grayi*, this sequence was compared to the *Pipile cumanensis grayi* voucher data and only 1 point mutation was found to occur at the final base of the GenBank sequencing. It can therefore be concluded that the GenBank *Pipile cumanensis grayi* data is correctly assigned to the species and the single base may either be an error of sequencing as it is placed at the end of the sequence data or is a true SNP. This can be accepted as DNA evidence for species origin as 1 point mutation within 400 bases is generally accepted as intra-species variation (Linacre, 2009) here seen within 660 bases.

Table 4.5: Point mutations in *Pipile pipile* and *Pipile cumanensis grayi* Cyt *b* sequence alignment.

Sequence Comparison	SNPs	Base position of mutation in sequencing alignment								
Reference *Pipile pipile* Vs *Pipile pipile* AY367106	9	/	/	/	/	/	/	/	/	471
Reference *Pipile pipile* Vs voucher *Pipile cumanensis grayi*	17	30	183	234	294	348	363	399	435	471
Voucher *Pipile cumanensis grayi* Vs *Pipile pipile* AY367106	0	/	/	/	/	/	/	/	/	/

Table 4.5 cont… Point mutations in *Pipile pipile* and *Pipile cumanensis grayi* Cyt *b* sequence alignment.

Sequence Comparison	SNPs	Base position of mutation in sequencing alignment							
Reference *Pipile pipile* Vs *Pipile pipile* AY367106	9	501	736	807	858	921	948	961	1138
Reference *Pipile pipile* Vs voucher *Pipile cumanensis grayi*	17	501	736	807	858	921	948	961	1138
Voucher *Pipile cumanensis grayi* Vs *Pipile pipile* AY367106	0	/	/	/	/	/	/	/	/

Summary of sequencing alignment of Cyt *b* with the base position of the point mutations which occurred. The point mutations in the comparison of the reference *Pipile pipile* sample to GenBank *Pipile pipile* data occur at the same positions as within the comparison of the reference *Pipile pipile* sample with the voucher specimen of *Pipile cumanensis grayi*. This provides evidence for the incorrect species assignment of the GenBank data.

4.6.3 ND2 Pairwise comparisons

To further investigate the relationship between the previously aligned sequences, pairwise comparisons were performed using all available genetic data for *Pipile*. Pairwise comparisons included data from the reference *Pipile pipile* sample and voucher specimen of *Pipile cumanensis grayi* as well as data for all other Piping guan species available on GenBank. Pairwise calculations were performed using the Kimura 2-Parameter (K2P) model as this is the conventional method for species comparison. Because the sequences on GenBank do not overlap, a combined analysis of ND2 could not be performed. An initial pairwise comparison using 441bp of the ND2 gene can be seen in table 4.6.

Table 4.6: Pairwise comparison (below diagonal) and nucleotide substitutions (above diagonal) within 441bp of ND2 *Pipile* data.

Sample	1	2	3	4	5	6
1. *Reference P. pipile*	-	5	5	8	7	9
2. *P. pipile*	0.011	-	0	3	4	10
3. *Voucher P. c. grayi*	0.011	0.000	-	3	4	10
4. *P. cumanensis*	0.018	0.007	0.007	-	5	13
5. *P. cujubi*	0.016	0.009	0.009	0.011	-	12
6. *P. jacutinga*	0.021	0.023	0.023	0.030	0.028	-

Average pairwise distance of 0.015 with a nucleotide diversity (π) of 0.014

Table 4.6 highlights the conservation of sequence data between the voucher specimen of *Pipile cumanensis grayi* and the GenBank *Pipile pipile* data. A pairwise distance of 0.011 was evident between the reference *Pipile pipile* data when compared with GenBank *Pipile pipile* and voucher *Pipile cumanensis grayi,* indicating the GenBank data to be *Pipile cumanensis grayi*. A variation of 0.007 was evident between *Pipile cumanensis* and *Pipile cumanensis grayi* (as well as GenBank *Pipile pipile* suspected to be *Pipile cumanensis grayi*) which would be expected in a species/sub-species comparison.

The second ND2 comparison seen in table 4.7 shows the pairwise differences when 451bp of the ND2 gene were aligned. The same pattern is evident regarding the GenBank *Pipile pipile* data with no variation seen when compared with the voucher *Pipile cumanensis grayi* data.

Table 4.7: Pairwise comparison (below diagonal) and nucleotide substitutions (above diagonal) within 451bp of ND2 *Pipile* data.

Sample	1	2	3	4	5	6
1. *Reference P. pipile*	-	7	7	6	5	8
2. *P. pipile*	0.016	-	0	1	4	11
3. *Voucher P. c. grayi*	0.016	0.000	-	1	4	11
4. *P. cumanensis*	0.013	0.002	0.002	-	3	10
5. *P. cujubi*	0.011	0.009	0.009	0.007	-	7
6. *P. jacutinga*	0.018	0.025	0.025	0.023	0.016	-

Average pairwise distance of 0.013 with a nucleotide diversity (π) of 0.012

4.6.4 Cyt b *pairwise comparisons*

One set of Cyt *b* sequence data is currently available on GenBank for *Pipile pipile* allowing a single comparison to be performed (see table 4.8). In addition, GenBank data is also available for *Pipile cumanensis grayi* and was included as a comparator not only for *Pipile pipile,* but also for the voucher *Pipile cumanensis grayi* data.

Table 4.8: Pairwise comparison (below diagonal) and nucleotide substitutions (above diagonal) within 353bp of Cyt *b Pipile* data.

Sample	1	2	3	4	5	6	7
1.Reference *P. pipile*	-	3	3	4	3	3	5
2. *P. pipile*	0.009	-	0	1	2	2	6
3. *Voucher P.c. grayi*	0.009	0.000	-	1	2	2	6
4. *P. c. grayi*	0.011	0.003	0.003	-	3	3	7
5. *P. cumanensis*	0.009	0.006	0.006	0.009	-	2	6
6. *P. cujubi*	0.009	0.006	0.006	0.009	0.006	-	6
7. *P. jacutinga*	0.014	0.017	0.017	0.020	0.017	0.017	-

Average pairwise distance of 0.010 with a nucleotide diversity (π) of 0.009

Following the sequence comparison it is evident that overall, cyt *b* is less variant than ND2 having an average pairwise distance of 0.010. Although 892bp of ND2 compared with 353bp of cyt *b* were aligned, it would be expected that average variation would be greater in cyt *b* given that it is variation within this gene that is currently used for species identification in legal cases. However, overall variation was found to be 0.8% in the cyt *b* gene compared with 1.3% in the ND2 gene. The patterns of inter-species similarity concur with that seen in the comparisons of the ND2 data, providing further evidence of misclassification of the GenBank *Pipile pipile* data. The highest pairwise distances in each comparison originate from *Pipile jacutinga* sequence data with *Pipile pipile* showing the second most variant data. This suggests that the placement of the *Pipile pipile* on the species phylogeny may alter when analysed with data from the current study.

4.6.5 Outcome of sequence alignments and pairwise comparisons

With regards to *Pipile cumanensis grayi* sequence data, that which was derived from GenBank was removed from further analysis as data from a voucher specimen was obtained in the present study. The single SNP within the GenBank data located at the final base of sequencing also reduced confidence in this respect. As this point mutation could not be verified by study of an

electropherogram, it could not be confirmed whether this was a true SNP. In addition, as the pairwise distances evident for Piping guans are low, a single incorrect base would apply an inaccurate weighting to the data in further analysis, known as ascertainment bias (Garvin et al., 2010).

To further examine the GenBank entries, the sequence data for cyt *b* of both *Pipile pipile* and *Pipile cumanensis grayi* (AY659797 and AY367106) were aligned. The sequences overlapped at 352bp of the 660bp alignment and were 100% identical, providing further evidence for the same species origin. The GenBank data for *Pipile pipile* was therefore not included in further analyses. For the purpose of this study, when data was required for representation, the sequence data which was derived from the reference sample of *Pipile pipile* was used for phylogenetic analysis. Because all other Piping guan species are morphologically dissimilar, mainly in the colouration of the dewlap, the species identification of all other data available on GenBank has a higher level of confidence and can therefore be included in further analyses. Although the previous comparisons give some indication to the placement of *Pipile pipile* and *Pipile cumanensis grayi* within the phylogeny based on K2P distances, this could not be inferred without a revised phylogenetic analysis.

4.7 Phylogenetic re-assessment

Pipile cumanensis grayi has never formally undergone phylogenetic assessment using sequence data. However, if the data used in the phylogeny created by Grau et al., (2005) for *Pipile pipile* is in fact *Pipile cumanensis grayi*, this branch can be re-classified on the original tree. If *Pipile cumanensis grayi* is inserted where *Pipile pipile* was placed (see figure 4.5) it can be seen that this species pairs with *Pipile cumanensis*, providing genetic evidence of the sub-species classification. What is also apparent is that the branching of *Pipile pipile* is now unknown and therefore requires re-assessment.

Fig. 4.5: Edited version of phylogeny constructed by Grau et al., (2005).

Sequence data of ND2 and cyt *b* obtained in the present study for *Pipile pipile* and *Pipile cumanensis grayi* were subsequently combined with data currently available on GenBank for *Pipile cujubi, Pipile cumanensis, Pipile jacutinga* with *Crax rubra* as an out-group to create an alignment of 1884bp. This data was used to construct a revised phylogeny of Piping guans using jModelTest and MEGA4 and the first true placement of *Pipile pipile* within a Piping guan phylogeny was calculated. The tree obtained in the current study can be seen in figure 4.6.

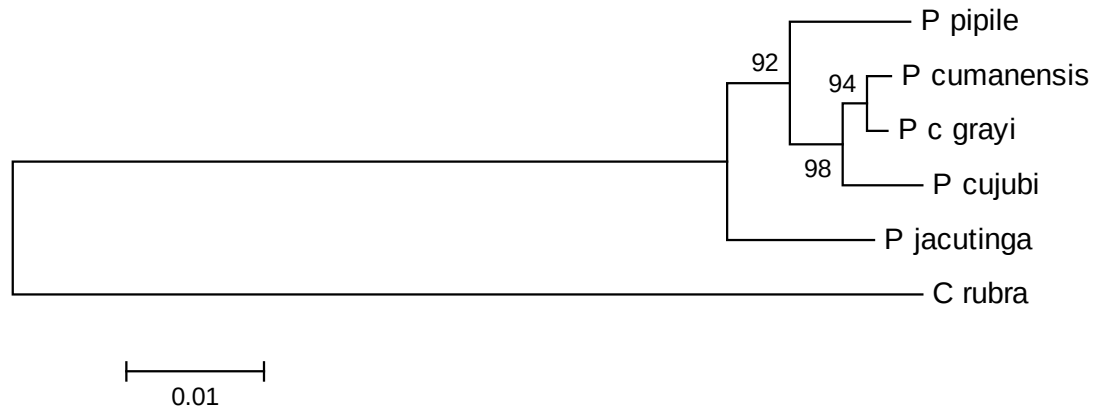


Fig. 4.6: Phylogenetic re-assessment of Pipile pipile *using combined analysis of cyt* b *and ND2 sequences. The tree was constructed using the neighbour joining method with Tamura-Nei's model of sequence evolution. Bootstrap values of an interior node test with 1000 replicates are placed on the topology.*

In the original phylogenetic analysis by Grau et al., (2005) sequence data obtained from the mitochondrial D-loop was also used to construct the phylogeny. However, D-loop data was omitted from the current study as this region is non-coding and usually has a much higher mutation rate. This data is normally used because the mutation rate assists the separation of closely related species; however Pereira et al., (2004) have shown that the D-loop of Cracids appears to evolve at a slower rate than cyt *b* and ND2. Because the data used by Pereira et al., (2004) for *Pipile pipile* has been misclassified these findings cannot be assumed for the species. Though it would be expected that the rate of evolution would be consistent throughout the Piping guans, without conclusive data the D-loop region was excluded from the current analysis.

Discussion

4.8 GenBank Pipile *datasets*

All current GenBank entries for *Pipile pipile* (AY367106, AY367100, AY367094 and additionally D-loop data AY145320) were obtained from the same specimen held at Granja 'La Siberia' in Mexico. From intra-species analysis of *Pipile pipile* conducted in the current study with reference data obtained from a true island bird it has been found that the previously published *Pipile pipile* data has been incorrectly classified. The bird held at the private aviary in Mexico belongs to the sub-species *Pipile cumanensis grayi* and therefore all publically available genetic data for the Trinidad Piping guan appears to be inaccurate.

The identification of this misclassified species supports Peterson et al., (2007) regarding the use of voucher specimens and the arguments of Olsson et al., (2008) that additional information should be collected from non-voucher specimens to support the classification. The collection of supporting data can sometimes avoid the misidentification of a reference sample, in this case tracing the origin of captive birds. Despite assurance to the authors that the species were bred from pure parentage (Pereira & Baker, 2004) analysis in the current study has shown this to be incorrect. Due to the vast similarities between *Pipile pipile* and *Pipile cumanensis grayi* this is most likely to have been unknown by the handlers.

4.8.1 Comparison of published phylogenies with that of the current study

The phylogenetic tree constructed in the current study places *Pipile pipile* closer to the root of the phylogeny than the previous published assessment. Bootstrap support of 90 or higher is generally accepted to show the 'correct' node making the results of the current study highly supported. When comparing the phylogeny devised in the current study (figure 4.6) to that of Grau et al., (2005) (see chapter 1, figure 1.2), patterns of similarity can be identified such as the placement of *Pipile pipile* in Grau's topology being substituted in the current study by *Pipile cumanensis grayi*. The divergence time of *Pipile pipile* is therefore greater than that calculated by Grau et al., (2005) with the previous

calculation now relating to *Pipile cumanensis grayi* (0.4 - 1.6MYA). As *Pipile pipile* appears to have been previously misrepresented, the placement seen in figure 4.6 is the first true phylogenetic assessment of the species.

4.8.2 Bio-geographical implications

Since publication of the previous phylogenetic analysis of Piping guans by Grau et al., (2005) which stated that Trinidad was last connected to mainland 11,000 years ago; new research has shown that this dating could be incorrect. It is now believed that Trinidad may have separated from the mainland as recently as 1500 years ago (Van den Eynden, Oatham & Johnson, 2008) after the breaking of a land bridge from the South-Western Peninsula (Kenny, 2008).

Due to the recent land bridge connection, genetic diversity from other mainland species would be expected to be low as in terms of speciation events, island species become distinct through isolation from the original population. For the Trinidad Piping guan this isolation has only occurred in the past 1500 years. The high level of mutation from other Piping guan species (shown through pairwise comparisons) would therefore demonstrate an early genetic divergence. This level of diversity is improbable within 1500 years and if occurring because of isolation, may have been demonstrated by the phylogeny. This provides support for the genetic divergence seen in the remaining population of the Trinidad Piping guan to have been caused by the immigration of individuals from the mainland prior to Trinidad's separation. However, further research of *Pipile piple* and *Pipile cumanensis grayi* mitochondrial haplotypes is required to investigate this hypothesis. This is in concurrence with Grau et al., (2005) where inconsistency in molecular dating was explained by the possibility of dispersal from the mainland.

After the point at which Trinidad became an island, the remaining population (the founder for the species) would have become geographically isolated. As flight is not sustained within the Piping guans, migration and therefore new alleles cannot be introduced creating a loss of genetic drift and reducing variant haplotypes. The effects of hunting may therefore have removed genetically

important individuals from the population and caused a bottleneck of genetic variance. The loss of populations in the southern and central regions may also have had dire effects on diversity, possibly introducing an issue of inbreeding. What is apparent from the current study (due to the phylogenetic placement of the species) is the need for further genetic analysis of Piping guans. Numerous individuals from all species require study (at a nuclear as well as mitochondrial level) to identify variant alleles and clarify that the mitochondrial haplotype observed in the Trinidad Piping guan does not appear in other species. As only one individual of most species of Piping guan have been studied, the division of the currently recognised species by genetic analysis (especially that of *Pipile pipile*) may have occurred by selecting divergent mitochondrial haplotypes by chance. In the current study, similarity was found between the bird held in captivity in Mexico thought to be *Pipile pipile* and of *Pipile cumanensis grayi*. Based on current evidence the captive bird has been misidentified, although research should be conducted on *Pipile cumanensis grayi* mtDNA haplotypes to ensure that the sub-species does not contain the Trinidad haplotype. The implications of finding the Trinidad haplotype within mainland birds would throw species classification and current conservation strategies into question. If future work demonstrated that genetic variation was not great enough to distinguish the Trinidad Piping guan as a species this would have dramatic effects on current researchers. This finding would be positive in that the species would no longer be classed as critically endangered but Trinidad would then lose its only endemic bird and hunting may increase. Should this happen, the current population could disappear and although reintroduction from mainland birds may be possible this would be expensive and perhaps inefficient if hunting were to continue. The true inter-species relatedness may therefore become clearer and enable a greater picture of evolutionary history with further genetic study.

4.8.3 The placement of **Pipile cujubi**

A further unexpected outcome of this re-assessed phylogeny was the placement of *Pipile cujubi* between *Pipile pipile* and *Pipile cumanensis grayi*. Because of

the genetic and morphological similarity of the species, it was expected that these species would lie on adjacent branches. This demonstrates the importance of inferring species relationships from phylogenies, not simply estimating divergence from genetic data, such as the previously calculated pairwise comparisons or morphological characteristics. Though pairwise comparisons are useful for calculating the level of similarity between sequences, this can be subject to statistical problems. Simply comparing species SNPs may identify variation which when calculated with a model of evolution does not significantly affect the tree generated. The results of the calculated phylogeny therefore indicate that *Pipile cujubi* is more closely related to *Pipile pipile* than *Pipile cumanensis* or the sub-species *Pipile cumanensis grayi*.

4.8.4 Merging Pipile *within* Aburria

The decision to include *Pipile* within *Aburria* (Delacour & Amadon, 1973; Pereira, 2000; Grau et al. 2005; Frank-Hoeflich, et al., 2007) as discussed in chapter 1 was examined by constructing a maximum parsimonious tree using the nearest neighbour interchange method with 10 replicates. GenBank data for *Aburria aburri* (AY140740 and AY354489) was included in the original 1884bp dataset and the resulting tree can be seen in figure 4.7. The branching of this species within the Piping guans supports Delacour & Amadon, 1973; Pereira, 2000; Grau et al. 2005 and Frank-Hoeflich, et al., 2007 who state that *Aburria* should merge with *Pipile*.

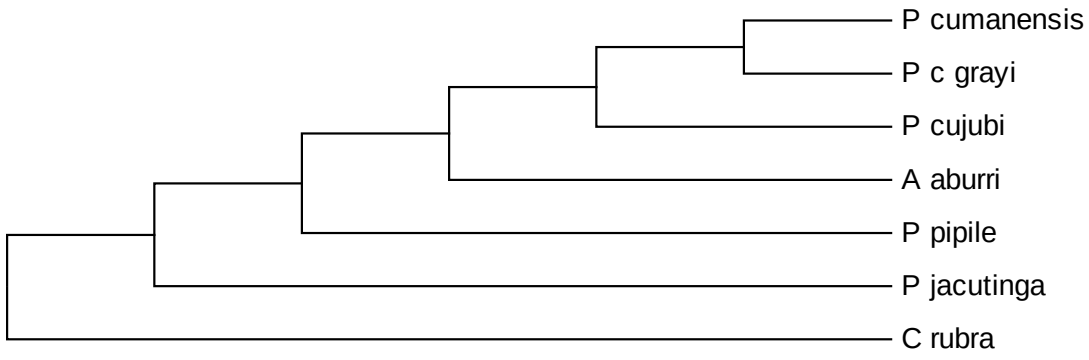


Fig. 4.7: Maximum parsimonious topology of Pipile *and* Aburria *data calculated using nearest neighbour interchange with 10 replicates.*

Comparison of this maximum parsimonious tree to that constructed by Grau et al., (2005) clearly shows the branching characteristics to be essentially identical, changing only at the inclusion of *Pipile pipile* between *Pipile jacutinga* and *Aburria aburri*. The analysis therefore concurs with previous findings but with the important alteration of the placement of *Pipile pipile.*

To further examine this merge of *Aburria* and *Pipile*, a neighbour joining tree was drawn using the Tamura-Nei model of evolution, this tree can be seen in figure 4.8.

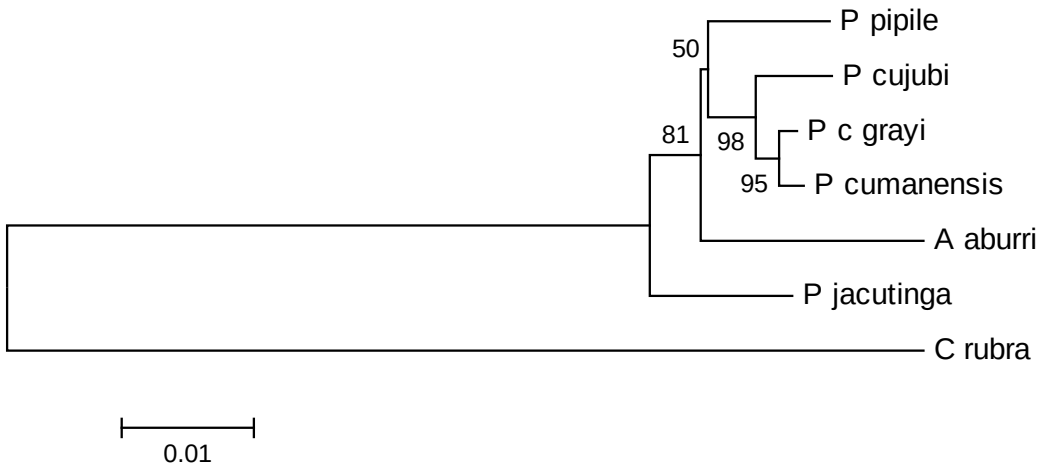


Fig. 4.8: Neighbour joining tree using Tamura-Nei's model of sequence evolution. Interior node bootstrap values are placed on the topology.

When compared to the maximum parsimonious topology in figure 4.7, it can be seen that *Aburria aburri* now appears between *Pipile jacutinga* and *Pipile pipile* but the bootstrap support is seen to decline when this species is included. Although grouping with the Piping guans and therefore supporting the merge, the low nodal support demonstrates that further investigation is required. Because studies have not been conducted on voucher specimens, Piping guans will still be referenced as '*Pipile'* throughout this work, in concurrence with IUCN, IOC and Bird Life classification.

4.9 Conclusion

Sequence comparisons presented for ND2 and Cyt *b* regions produced a 100% similarity match between GenBank *Pipile pipile* and voucher *Pipile cumanensis grayi* obtained in the current study. As such it can be inferred that the bird held at the private aviary in Mexico originally classified as *Pipile pipile* has been wrongly identified and is the common sub-species *Pipile cumanensis grayi.* Due to this misidentification, the current study is therefore the first genetic analysis of the Trinidad Piping guan from which seminal data has been derived.

The data obtained in the present study have for the first time allowed the construction of a phylogeny of Piping guans to include a representative of *Pipile pipile* using a Trinidad sample and voucher specimen of *Pipile cumanensis grayi.* Phylogenetic calculations have altered the branching pattern for *Pipile pipile*, which has implications for the evolutionary history of the species. It is often seen that island species are more recently diverged due to isolation from the original population. However, because of the recent land bridge connection to South America, the species does not appear to conform to conventional island species characteristics. In addition, the placement of *Pipile cujubi* between *Pipile pipile* and *Pipile cumanensis grayi* implies that morphological similarities are the result of homology rather than an inference of relatedness.

Data derived in the current study have also demonstrated that more research is required regarding the merge of *Pipile* with *Aburria* due to low nodal support and the lack of voucher representatives. Further genetic work is also required to support the current species classification of Piping guans by examination of mitochondrial haplotypes and microsatellites.

As the sequence information available on GenBank for *Pipile pipile* appears to originate from *Pipile cumanensis grayi*, the use of this data for species identification would have provided a misidentification. It is therefore necessary for genetic data and methodologies to be established for identification of the species for use in field studies and instances of forensic study.

Chapter 5

The development of species detection and genetic identification methods for use in field studies

Introduction

5.1 The applications of genetic identification

Identification of the Trinidad Piping guan is currently reliant upon sightings in the field and the assessment of morphological characteristics. This causes problems when estimating population size by distance sampling (Keane et al., 2005) and would be an issue in the identification of wildmeats (and possibly museum samples) if the full carcass was not available for examination. Previous chapters have shown that the Trinidad Piping guan has been subjected to hunting pressures (Brooks, 1998) and although this threat has diminished in recent years, evidence suggests that occasional hunting does still occur. Wildmeats sold out of season are an issue in Trinidad (Francis, 2011) and consumption within season is common occurrence (see figure 5.1). However, it is suspected that the Trinidad Piping guan is not a part of trade and that any slaughtered birds are consumed by hunters and their families.

Prior to the current study, genetic identification of the Trinidad Piping guan has not been possible and as such, meats or derivatives suspected to originate from the species could not be positively identified. The ability to determine remains may therefore act as a deterrent if unknown meats can be confiscated, identified and possible action taken against offending hunters. Not only would this identification method assist hunting regulations but it may also benefit ecological studies by locating areas of habitation using trace evidence such as feathers. In the Pawi Species Recovery Strategy (Nelson et al., 2011), action 7.1 states that a survey is required to improve current knowledge of species distribution and population numbers. In additon, it is stated in the introduction of this strategy that recent sightings have occurred in the South-Eastern forest regions of Moruga and Trinity Hills. By collecting non-invasive feather samples for species

identification, it may be possible to confirm these sightings without observation and therefore improve knowledge of the current distribution of the species. This work would therefore aid the completion of point 1.1 '*Conduct a distribution and population survey throughout all known and potential existing habitat'*. Feathers could also be collected by members of rural communities which helps realise point 1.3 '*Develop a participatory monitoring programme that both engages the communities in Pawi areas and provides meaningful data*…' without the need for extensive training or specific excursions (Nelson et al., 2011).

A further application for genetic identification is the definitive species classification of captive birds. Identifying the Trinidad Piping guan from its most morphologically similar species, *Pipile cumanensis grayi,* is a difficult task of which few professionals are able to conduct. Though some features can differentiate the species, these are minor colour variations (Delacour, Amadon, del Hoyo & Motis, 2004) which can be difficult to distinguish. Identifying captive-bred birds is therefore important should re-introduction programmes become necessary in the future. The feasibility of captive breeding programmes within Trinidad are currently being discussed but conservation of the species and its habitat are the main priority (Nelson et al., 2011).

Because of the limited genetic data available for the species and the death of captive island birds, it would seem imperative for a method of identification to be established. This method may in turn assist conservation of the species, via distribution studies; ecological research studies through the location of habitat and species identification of hunting kills in wildlife investigations.

Fig. 5.1: Local notice in Trinidad in which 'wildmeat' is advertised
Photograph provided by Margaret Cooper

5.1.1 The suitability of wildlife forensic methods to the current study

The field of wildlife DNA forensics provides numerous approaches to species identification which can be applied to the current study. When the full carcass of an animal remains or species identifying characteristics are available, it may be possible for species classification to be conducted by a forensic veterinarian or similar professional by assessing distinctive morphological characteristics. However, if these features are indistinguishable or missing, as is often the case due to there removal by hunters (Linacre, 2009) identification by genetic analysis may be required. In addition, the identification of derivatives obtained in field studies can be difficult if species defining characteristics are not present.

Ideally, identification methods should be established using a sample taken from a voucher specimen of the species as there can be no cause for suspicion of origin of this sample. However, as discussed in chapter 4, this voucher may not always be available and a reference sample must be obtained in its place. The

selected sample must then undergo genetic analysis by sequencing mitochondrial gene regions and identifying variations which distinguish it from its most closely related relatives (Linacre et al., 2010). Though these relatives may be distinguishable by eye, little genetic variation may be observed if the species have only recently diverged. The tests for identification must therefore be sensitive enough to overcome any genetic similarity between samples. Genetic diversity is therefore used to establish methods of identification either directly by sequencing informative regions or indirectly using techniques such as PCR.

The standard approach to species identification is to use sequence information from the mitochondrial genome as the higher mutation rate assists identification through inter-species variation. Numerous methods are available for genetic identification and the chosen technique for an investigation will depend upon the species of study and sample availability.

5.1.2 Forensic and field sample similarities

Material requiring identification (whether through forensic casework or research samples) is often of inferior quality and may have undergone degradation. This can occur due to the length of time a sample remains in the field and the conditions it is exposed to. Due to the use of inferior samples, it is necessary that the methods established to conduct species identification are highly sensitive and take possible restrictions such as degradation into account by examining small regions of the mitochondrial genome. By using samples in the current study which are typical to that obtained in wildlife crime investigations and research studies, the established methods are therefore known to allow detection from inferior samples. Genetic identification methods which are determined with the use of DNA extractions from optimal samples, such as blood, may have problems with sensitivity when applied to remotely collected samples or trace evidence.

5.2 Identification by databases

Originally, species were identified by the same methods as human identification using nDNA methods. This included genetic fingerprinting and immunological procedures which would often only provide identification down to the species family level (Parson, Pegoraro, Niederstätter, Föger & Steinlechner, 2000). In later years it became apparent that these methodologies were unnecessary and that variation within one gene of the mitochondrial genome would provide enough unique sequence data for species identification when compared to a database of genetic information. Identification can be performed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) available on the NCBI website which compares target sequence data to GenBank entries. In August 2009, the number of sequences deposited on GenBank reached 108 million (Linacre & Tobe, 2011) and for this reason when searching the database, researchers are able to select certain datasets to perform comparisons. BLAST compares the sequenced fragment to a dataset and produces similarity scores for the one hundred most closely matched sequences. Species identification therefore occurs by the obtainment of a high similarity score (99-100%). A 100% match is often found from a database search which in combination with a pairing of less than 97% with another species provides a high level of confidence in the identification, depending on the length of the sequence compared. The method of species identification by database search has been validated for use and approved by the International Society for Forensic Genetics (ISFG) (Ogden, Dawnay & McEwing, 2009).

Due to the current study identifying misclassification of current GenBank entries, identification in the current study was performed by comparison to the reference material obtained from the captive Trinidad Piping guan. When studying closely related species, as in the current study, SNPs can be used to discriminate between species rather than relying on a percentage match cut-off point. This can improve the confidence level of the species assignment (Linacre, 2008) but species defining SNPs cannot be identified without thorough study. Currently, there are no standard tests to identify species, nor a consensus on how many

SNPs define a species (Tobe & Linacre, 2007; Linacre, 2009) however genes have been validated for identification purposes.

5.2.1 Universal Cytochrome b *primers*

Because of the volume of genetic data and wide taxonomic representation of cyt *b,* with 8000 sequences being available online a decade ago (Parson et al., 2000) this gene has become the standard for use in species identification. In 2003, the use of this gene was validated for species identification by nucleotide sequencing following Technical Working Group on DNA Analysis Methods (TWGDAM) methods (Branicki, Kupiec & Pawlowski). Prior to validation, cyt *b* was demonstrated in a number of studies as a successful identification marker (Smith & Patton, 1991; Carr & Marshall, 1991; Parson et al. 2000; Hsieh et al. 2001; Verma & Singh, 2003).

If an unknown sample is not suspected to have become contaminated through human contact or by other wildlife DNA, universal primers will prove the most informative initial analysis. These primers are created by aligning genetic sequences of many different species from a range of genus's and identifying conserved regions which are then used to create primers. Universal primers can therefore amplify a fragment of DNA for species identification by sequence comparison. The universal cyt *b* primers designed by Kocher et al., (1989) and Irwin, Kocher, & Wilson (1991) have been used in numerous identification studies due to their applicability to a wide variety of species (Hsieh et al., 2001; Wong, Wang, But & Shaw, 2004; Tsai et al., 2007). The continued use of these primer sets therefore increases the volume and variety of genetic information available for comparison of this gene region. Alternate primer sets for species identification have subsequently been designed (Verma & Singh, 2003) which have shown to be successful in a number of wildlife crime cases (Sahajpal & Goyal, 2010; Peppin et al., 2008). In addition, to improve reference data currently available on GenBank, whole gene universal primer sets have been designed (Naidu, Fitak, Mungula-Vega, Culver, 2011).

5.2.2 Drawbacks of universal methodologies

The use of universal primers becomes problematic when a sample becomes contaminated. This is a potential issue in the current study due to the use of field samples which may possibly become contaminated prior to collection. Because universal primers are designed for the purpose of amplifying DNA from a high number of species, using these primers on a mixed sample will most likely amplify DNA from all species which are present. If sequence analysis is then conducted, a mixed and unreadable electropherogram is produced and the data obtained cannot be used for comparative purposes. Though there are techniques available to estimate human contamination within a sample and calculate the concentration of non-human mammalian DNA present (Tobe & Linacre, 2008) methodologies have not been established for mixed wildlife DNA samples. Universal primers therefore cannot be used for sequencing with contaminated wildlife DNA without the additional process of cloning and SNP markers (Ogden, 2011). The identification of species present within a mixed sample therefore requires the use of species specific primers or restriction fragment length polymorphisms (RFLPs).

5.2.3 Species Specific Primers

Unlike universal primers, species specific primers require high levels of variation and must contain SNPs which are seen only in the target species. Their design requires a point mutation at the 3' end of the primer to allow extension to occur only in the target species DNA. These primers are created in the same way as universal sets by aligning sequence data, but regions of variation rather than conservation are identified (Wan & Fang, 2003). If DNA of the target species obtained in an investigation is of low quantity because of the sample type or degradation, this primer specificity improves the likelihood of amplification. Primers are therefore sensitive enough to extract only the target species DNA within a mixture to provide clear sequencing (Tobe & Linacre, 2007; Tobe & Linacre, 2009). Species primers can also be used within the second round of nested PCR to enable identification of low quantity DNA (Li, Hua & Yeh, 2010).

Previously, species primers have been used to detect the origin of materials such as faeces, (Dalén, Götherström & Angerbjörn, 2004; Michalski et al., 2011) determine predated animals within faeces (Vestheim, Edvardsen & Kaartvedt, 2005) and identify species within the animal itself, such as that consumed by insects (Cassel-Lundhagen, Öberg, Högfeldt, & Ekbom, 2009). These primers therefore also have applications within research, providing rapid identification methods of remotely collected non-invasive genetic samples. However, if primers are not validated, nucleotide sequencing must also be conducted in order to confirm species identification if used in legal cases rather than field work. Species specific primers have been designed for a number of endangered species and those which are commonly the target of wildlife trade (Yan et al., 2005; Hsieh et al., 2006). However, obtaining DNA samples in order to create these methods can prove problematic as many endangered species are difficult to locate and the collection of samples, even non-invasive, can be challenging.

The use of this method in the current study would allow the rapid elimination of confusion species found in Trinidad, such as vultures, in the identification of field samples. This therefore reduces the cost of an investigation as sequencing is not required. In addition, universal and species specific primer sets can be utilised further by performing analysis on the DNA fragments they amplify. Restriction endonucleases can identify species by creating specific restriction digests, even within mixed or degraded samples (Bravi et al., 2004) as the banding patterns observed after digestion with enzymes can be species specific and therefore enable identification.

5.2.4 Restriction Fragment Length Polymorphisms

Restriction endonucleases are enzymes isolated from bacteria which identify a specific site of 4-, 5-, 6- or 8- base sequences and digest at the recognition site within the genetic sequence (Cronin, Palmisciano, Vyse & Cameron, 1991). There are many enzymes available for the digestion of a DNA strand and choosing the correct enzyme to allow detection is definitive to the success of the method. RFLPs can be utilised when genetic variation is low and species

primers are not specific enough to identify between species within a genus. In this instance, a single SNP if occurring at a restriction site can separate closely related species by expressing a different number of restriction digests with a specific enzyme. Large fragments or whole genome sequences cannot be analysed with endonucleases as there are too many restriction sites present within the sequence to present a clear result. If the frequency of sites is too high, separation of fragments will not be possible and a smear will be present after electrophoresis (Parker, Snow, Schug, Booton & Fuerst, 1998).

Not only can this method be used on pure DNA amplifications but it can also be performed on contaminated samples amplified by universal primers provided the banding pattern for the target species is known. Where genetic information is available but species detection methods have not yet been established, sequence data from GenBank can be analysed using online software such as Restriction Mapper (Blaiklock, 2002; Brakmann, 2002). This output identifies enzymes which create different banding patterns between species, through the presence or absence of restriction sites. The greater the number of enzymes which are used to conclude species detection, the more robust the method as intra-species variation could lead to erroneous results (Chen, Liu, & Yao, 2010). RFLP analysis has also been shown to effectively separate sub-species where other methods such as species specific primers fail (Ferreira, Martins, Ditchfield & Morgante, 2005).

5.2.5 Nucleotide Sequencing

The previous methods of species primers and RFLPs, though accepted methodologies within forensic genetics, are applied within the context of species detection rather than identification. Species identification must be confirmed by the validated technique of Forensically Informative Nucleotide Sequencing (FINS) within regions of validated genes if required for legal cases (Bartlett & Davidson, 1992; Blanco, Pèrez-Martin & Sotelo, 2008).

In comparative sequence analysis, a reference sample of the species must be available in order for sequence similarities to have any validity. If species origin

of a sample can be speculated, the data obtained can be compared to sequences from the target species to produce identification. However, if origin is unknown, sequence data obtained can be subjected to a full database search using BLAST. As previously mentioned, although there is no overruling consensus of definitive species identification, it is generally accepted that there is an allowance of 1 in 400 bases of intra-species variation (Linacre, 2009).

5.2.6 Species identification utilising nuclear DNA

Human genetic profiling provides strong evidence in court due to the high statistically probability that data are unique to the individual; however this is not the case for all species. The method relies on genetic diversity and defining an individual becomes more difficult as population size and gene flow decrease.

This method is used when individual rather than species identification is required or population studies are being conducted, though some species specific microsatellites have been developed (Abdelkrim, Robertson, Stanton & Gemmell, 2009). Because microsatellites look for variation within the genome it is sometimes unfortunate that those most in need of genetic investigation, such as endangered species, lack this diversity. Because of small population numbers, the genetic variation in some endangered species may be too low to provide distinction from sister species. It is also likely that if identification was possible, a much higher number of markers would be required for confidence in the identification (Piggott & Taylor, 2003).

Because of the estimated low population numbers of the Trinidad Piping guan, genetic identification was performed purely by the use of mtDNA. Although this would only result in the identification of species rather than individual, all members of this species originate from the same critically endangered population and therefore individual identification was deemed unnecessary.

5.3 Gene and species selection

As discussed in chapter 4, the genetic variability of ND2 has been observed to be greater than within cyt *b*, assisting the similarity score of compared species. This higher level of genetic variability increases the chance of distinction between *Pipile pipile* and other Piping guan species. However, as ND2 has not been validated for species identification, methodologies using the cyt *b* gene were also required should these techniques ever be necessary for the investigation of evidence in legal cases rather than fieldwork.

It is known that variation becomes more abundant in distantly related species; therefore creating methods which distinguish a species from its closest relatives are highly likely to separate it from all other species providing a sound method of detection. Taking into account morphological characteristics, *Pipile cumanensis grayi* was chosen as the species against which the established methods should be directly tested. Although the current study has shown that this is the not the closest relative of the Trinidad Piping guan, it is the species most likely to be misidentified by morphology. Identification of captive stock is therefore more likely to be required to separate these species than other members of the Piping guan family. As no other Piping guan species is found in Trinidad, the use of detection methods in field studies would require separation from confusion species rather than close relatives. In addition, as more data are available on GenBank for other Piping guan species, the methods established can undergo examination using downloaded sequence data.

5.3.1 Additional comparator species

The black vulture (*Coragyps atratus*) which is found in Trinidad was chosen as a comparator species for direct analysis as it is a possible confusion species, being of similar size and colour to that of the Trinidad Piping guan. Field samples such as feathers may therefore be difficult to identify and distinction between these morphologically similar species may be required more frequently than between that of other birds on the island. In addition, it is important that common domestic fowl are not amplified with species specific primers and

therefore feather samples of chicken (*Gallus gallus domesticus)* were acquired for direct testing. Also available for analysis were feather samples taken from *Crax blumenbachii* and *Crax fasciolata*, these species are relatives of the Piping guans and therefore provided a method of testing the specificity of the primers within Cracids. Using material obtained in the current study from the Emperor Valley zoo for *Pipile pipile* and voucher material from the University of São Paulo for *Pipile cumanensis grayi*, rapid detection methods were established which could be used in future studies of the Trinidad Piping guan using the principles of wildlife DNA forensics.

Methods

5.4 Developing species specific primers

Species primers are reliant on inter-species variation and therefore a gene region containing a high number of SNPs will be most likely to produce an informative marker which can distinguish between closely related species. The fewer the number of SNPs present, the more likely that closely related species will also be amplified with the designed primer set. Because species primers are also expected to be used in circumstances where DNA quality is sub-optimal, the size of the amplification must be appropriate for the investigation. The amplicon must therefore be of a suitable size (200-500bp) to avoid false negatives due to degraded DNA but contain enough informative sites should sequencing be required.

5.4.1 ND2 species specific primer design

Because of the previously identified high level of genetic variation in the ND2 gene, this gene was initially chosen for the development of species specific primers. ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997) was used to align 453bp of ND2 sequences from *Pipile pipile* and *Pipile cumanensis grayi* obtained in the present study with data available on GenBank for a number of Cracid species (for alignment see Appendices IV and table 5.1

for accession numbers of sequences used). Although the whole gene sequence was obtained for *Pipile pipile* and *Pipile cumanensis grayi* in the current study, a full gene comparison could not be conducted as the data are not available for the majority of Cracids registered on GenBank. Therefore, the largest possible gene fragment which incorporated the majority of Cracid species was used to conduct the comparison. The alignment identified many point mutations but by identifying those seen only in *Pipile pipile,* regions were identified for subsequent species primer design.

Table 5.1: Accession numbers used in ND2 species primer alignment

Species	Accession/Ref number
Pipile pipile	Pp_C.F.2006/ND2
Pipile cumanensis grayi	Ppg_V.F.2009/ND2
Pipile cumanensis	AY367093
Pipile cujubi	AY367092
Pipile jacutinga	AY140744
Aburria aburri	AY140740
Pauxi unicornis	AY141939
Pauxi pauxi	AY140750
Crax blumenbachii	AY140747
Crax rubra	AY141935
Crax globulosa	AY141934
Crax fasciolata	AY141933
Crax daubentoni	AY141932
Crax alector	AY141931
Crax alberti	AY141930
Mitu tomentosa	AY141938
Mitu salvini	AY141937
Mitu mitu	AY141936
Mitu tuberosa	AY140748
Oreophasis derbianus	AY140745
Ortalis canicollis	AY140746
Penelopina.nigra	AY140743
Chamaepetes goudotii	AY140741
Nothocrax urumutum	AY140749

5.4.2 Forward ND2 species primer

The forward 24mer species primer (LPawi1) lies at L5964 of the *Gallus gallus* mitochondrial genome X52392 (Desjardins & Morais, 1990) with a GC content of

45.8%. The clipped alignment of the primer region in all compared species can be seen in figure 5.2. This region was chosen as two point mutations occur near the 3' end of the primer improving specificity.

Because the final base at the 3' end of the primer falls on a 3rd base codon position and is therefore more vulnerable to mutation (Hartl & Jones, 2001) the initial species specific base (base 19 of the primer alignment) would be expected to assist the annealing of the primer. This first SNP seen within the sequence falls at a 1st base position and therefore mutation is restrained. Though it may have been beneficial for the initial SNP to be the final base of the forward species primer, removing the end of the primer sequence or pushing back the 5' end of the primer would have reduced the GC content. A reduction in GC content would lower the annealing temperature and therefore affect performance through non-specific binding, reducing the quantity of the required PCR product. The sequence prior to the 5' end of the species primer reads CCCTAAA; this base repetition is also not beneficial for 5' end Lstrand primer design as it creates problems with annealing and slipped strand mispairing.

5.4.3 Reverse ND2 species primer

Unfortunately, no SNP seen only in *Pipile pipile* occurred in the remainder of this sequence which was suitable for specific binding of a reverse primer. The reverse primer was therefore chosen to end at a SNP seen in three Cracid species, *Pipile pipile*, *Pipile jacutinga* and *Aburria aburri* (figure 5.3). The sequence of the primer region in *Pipile jacutinga* does however contain a single base variation from the designed primer at base 12, which may prevent annealing. Because of the specificity of the forward primer, amplification would only be expected to occur in *Pipile pipile*. The reverse primer fell at H6115 of the *Gallus gallus* mitochondrial genome (Desjardins & Morais, 1990) labelled HPawi1 and contained a GC content of 50%. The ND2 species primers therefore created an amplicon of 152bp within a region of limited base repeats. The creation of a primer set in which the PCR product is <200bp is of great assistance in cases where DNA may be degraded.

Fig 5.2: Alignment of the ND2 species-specific Lstrand primer 5' – 3' (LPawi1)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
P.pipile	G	C	A	G	C	C	C	T	A	A	T	A	C	T	C	A	C	C	T	T	A	C	T	T
P. cumanensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
P. cumanensis grayi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
P.cujubi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
P.jacutinga	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
A.aburri	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	C
P.unicornis	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
P.pauxi	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C. blumenbachii	.	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.rubra	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.globulosa	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.fasciolata	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.daubentoni	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.alector	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.alberti	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
M.tomentosa	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	C
M.salvini	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
M.mitu	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
M.tuberosa	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
O.derbianus	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
O.canicollis	.	.	.	A	.	A	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
P.nigra	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
C.goudotii	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C
N.urumutum	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C

This alignment demonstrates the specificity of the *Pipile pipile* species primer LPawi1. The first species SNP can be seen at base 19 which shows a transitional change from C to T. The second SNP occurs at the final base again seeing a transitional change from C to T in all species except *Ortalis canicollis*. The SNPs seen at bases 4, 6 7 and 19 should prevent the annealing of the primer to *Ortalis canicollis*

Fig 5.3: Alignment of ND2 species-specific Hstrand primer 5' – 3' (HPawi1)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
P.pipile	A	A	G	A	A	T	A	G	G	C	C	C	A	G	G	A	G	T	G	A	T	A	G	C
P. cumanensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
P. cumanensis grayi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
P.cujubi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
P.jacutinga	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
A.aburri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P.unicornis	.	.	.	.	.	.	.	.	.	.	T	A	.	.	A	.	.	.	.	.	.	.	.	T
P.pauxi	.	.	.	.	.	.	.	.	.	.	T	A	.	.	A	.	.	.	.	.	.	.	.	T
C. blumenbachii	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	C	.	.	T
C.rubra	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
C.globulosa	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
C.fasciolata	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
C.daubentoni	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
C.alector	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
C.alberti	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T
M.tomentosa	.	.	.	.	.	C	.	.	.	.	T	A	.	.	A	.	.	.	.	.	.	.	.	T
M.salvini	.	.	.	.	.	C	.	.	.	.	T	A	.	.	A	.	.	.	.	.	C	.	.	T
M.mitu	.	.	.	.	.	C	.	.	.	.	T	A	.	.	A	.	.	.	.	.	.	.	.	T
M.tuberosa	.	.	.	.	.	C	.	.	.	.	T	A	.	.	A	.	.	.	.	.	.	.	.	T
O.derbianus	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	A	.	T
O.canicollis	.	.	.	.	.	C	.	.	.	.	T	G	.	.	A	.	.	.	.	.	.	.	.	T
P.nigra	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T
C.goudotii	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	A	.	T
N.urumutum	.	.	.	.	.	C	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	T

In the reverse primer alignment, the final base of the primer shows a transitional change from C to T which occurs in *Pipile pipile, Pipile jacutinga* and *Aburria aburri. Pipile jacutinga* is however not 100% complementary, showing another transitional change from C to T at base 12 of the primer.

5.4.4 Cyt b *species specific primer design*

For the purpose of creating species specific primers within a validated region for species identification, primers were also developed within the cyt *b* gene. Data was obtained from GenBank (see table 5.2) to perform a comparative alignment of Cracids and identify species primers. Though not all entries contained full cyt *b* data, partial sequences were used to increase the number of species represented. Cyt *b* was found to contain fewer genetic variations than ND2, even though a larger number of bases were aligned and for a higher number of species (Appendices V).

5.4.5 Forward cyt b *species primer*

The forward 19mer species primer (LPawi2) which in the Desjardins & Morais (1990) *Gallus gallus* mitochondrial genome X52392 begins at L15108 has a GC content of 52.6%. Variation is seen within the primer between other Cracid species, however all aligned *Pipile* species were conserved at the primer region except the final base position, shown in figure 5.4. This base was conserved throughout all Cracid species except *Pipile pipile* and *Ortalis motmot*. Due to variation seen at two bases within the primer specificity is reduced in *Ortalis motmot*, helping to prevent annealing in this species.

5.4.6 Reverse cyt b *species primer*

The reverse primer was chosen to amplify a reasonably small region (271bp) whilst still obtaining enough data for species identification. Few SNPs were identified in *Pipile pipile* which differed from all aligned Cracid species and fell within a reasonable size range. A base was therefore chosen for the 3' end of the reverse primer which was unique in *Pipile pipile* to all other *Pipile* species but not all Cracids, as shown in figure 5.5. The specificity of the forward primer should be of assistance in the set only amplifying the DNA of the target species.

Two other point mutations occurred within the sequence alignment, unique in *Pipile pipile*, though some *Ortalis* species are not represented due to a lack of available sequence data. Using these SNPs would have produced a fragment of

500-700bp (depending if the designed forward primer was used or the initial reverse primer altered to be used as a forward primer) which is not beneficial due to the amplicon length. These SNPs occurred in the alignment at 807bp and 820bp (see Appendices V) in a region containing 5 repeating C bases, again creating the possibility of slipped strand mispairing. The primer (HPawi2) lies at H15379 of the Desjardins & Morais (1990) *Gallus gallus* mitochondrial genome X52392 with a GC content of 52.9%.

Table 5.2: Accession numbers used in cyt *b* species primer alignment

Species	Accession/Ref number
Aburria aburri	AF165466
Chamaepetes goudotii	AF165467
Penelopina nigra	AF165475
Chamaepetes unicolor	AY659796
Ortalis poliocephala	AY659784
Ortalis pantanalensis	AY659783
Ortalis guttala	AY659782
Ortalis ruficauda	AY659781
Ortalis garrula	AY659780
Ortalis leucogastra	AY659779
Ortalis motmot	AY659778
Ortalis canicollis	AF165472
Oreophasis derbianus	AF165471
Mitu tomentosa	AY141928
Mitu salvini	AY141927
Mitu tuberosa	AF165469
Mitu mitu	AY141926
Nothocrax urumutum	AF165470
Crax pauxi	AF068190
Crax rubra	AY141925
Crax globulosa	AY141924
Crax fasciolata	AY141923
Crax daubentoni	AY141922
Crax alector	AY141921
Pauxi gilliardi	AY659788
Pauxi unicornis	AY141929
Pauxi pauxi	AF165473
Pipile pipile	Pp_C.F.2006/cytb
Pipile cumanensis grayi	Ppg_V.F.2009/cytb
Pipile cumanensis grayi	AY659797
Pipile cujubi	AY659799
Pipile cumanensis	AY659798
Pipile cujubi	AY367104
Pipile cumanensis	AY367105
Pipile pipile	AY367106
Pipile jacutinga	AF165476

Fig 5.4: Alignment of cyt b *species-specific Lstrand primer 5' – 3' (LPawi2)*

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P.pipile	A	A	A	C	G	T	C	C	A	A	T	A	C	G	G	C	T	G	G
P. cumanensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P. cumanensis grayi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.cujubi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.jacutinga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
A.aburri	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.unicornis	G	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
P.pauxi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P. gilliardi	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
C.rubra	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A
C.globulosa	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A
C.fasciolata	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
C.daubentoni	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	A
C.alector	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	A
C. pauxi	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
M.tomentosa	G	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
M.salvini	G	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
M.mitu	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
M.tuberosa	G	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A
O. poliocephala	G	.	.	T	.	.	.	.	.	G	.	.	T	.	.	T	.	.	A
O. pantanalensis	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	T	.	.	A
O. guttala	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	T	.	.	A
O. ruficauda	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	T	.	.	A
O. garrula	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	T	.	.	A
O. leucogastra	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A
O. motmot	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.
O.derbianus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
O.canicollis	.	.	.	.	.	.	T	.	.	G	.	.	T	.	.	G	.	.	A
C. unicolor	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.nigra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
C.goudotii	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
N.urumutum	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A

Fig 5.5: Alignment of cyt b *species-specific Hstrand primer 5' – 3' (HPawi2)*

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
P.pipile	C	T	A	C	T	A	G	G	G	T	T	T	G	T	C	C	G
P. cumanensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P. cumanensis grayi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.cujubi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.jacutinga	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
A.aburri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P.unicornis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P.pauxi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
P. gilliardi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
C.rubra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C.globulosa	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
C.fasciolata	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
C.daubentoni	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C.alector	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
C. pauxi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
M.tomentosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M.salvini	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M.mitu	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
M.tuberosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. poliocephala	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. pantanalensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. guttala	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
O. ruficauda	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. garrula	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. leucogastra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. motmot	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
O.derbianus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O.canicollis	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
C. unicolor	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
P.nigra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
C.goudotii	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
N.urumutum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

5.5 Selection of ND2 restriction enzymes

RFLPs were selected by conducting a virtual digest with online software Restriction Mapper (Blaiklock, 2002; Brakmann, 2002) using sequencing results from amplification of ND2 using L5758. *EcoRV* was selected as the restriction site for this enzyme is not seen in the reference *Pipile pipile* sample but creates one digest in *Pipile cumanensis* and *Pipile cumanensis grayi*. The *BsaXI* enzyme produces two restriction digests (at one site) in the reference *Pipile pipile* sequence data but in almost all other *Pipile* species a second restriction site is present. Only one *Pipile* species was seen to match the digestion pattern of *Pipile pipile,* in data obtained from GenBank for *Pipile jacutinga.*

5.5.1 Selection of cyt b *restriction enzymes*

Virtual digests using Restriction Mapper were used also to identify RFLPs within cyt *b* sequence data obtained with L15311. From analysis of the virtual digest, very few enzymes produced a unique banding pattern in *Pipile pipile*. Only one enzyme was found to be suitable for analysis (*BsrDI*) which produced 1 digest in the reference *Pipile pipile* data but did not digest any other *Pipile* sequence. Table 5.3 lists all of the GenBank data used in Restriction Mapper for the restriction enzyme comparisons and table 5.4 shows the restriction sites of the chosen enzymes. Using all of the possible enzymes to perform this detection test increases the robustness of the method should intra-species variation occur in future studies.

Table 5.3: GenBank accession numbers of sequences used in RestrictionMapper comparison.

Species	Gene	Accession number
Pipile pipile	ND2	AY367094
Pipile cumanensis	ND2	AY367093
Pipile cujubi	ND2	AY367092
Pipile jacutinga	ND2	AY140744
Pipile pipile	Cyt *b*	AY367106
Pipile cumanensis	Cyt *b*	AY367105
Pipile cujubi	Cyt *b*	AY367104
Pipile jacutinga	Cyt *b*	AF165476

Table 5.4: Recognition sites of the restriction enzymes selected for species detection.

Restriction Enzyme	Gene	Recognition Sequence	Number of Restriction Digests				
			Pipile pipile	*Pipile cujubi*	*Pipile jacutinga*	*Pipile cumanensis*	*Pipile cumanensis grayi*
EcoRV	ND2	5'…GAT▼ATC…3' 3'…CTA▲TAG…5'	0	0	0	1	1
BsaXI	ND2	5'…▼$_{9}$(N)AC(N)$_5$CTCC(N)$_{10}$▼…3' 3'…▲$_{12}$(N)TG(N)$_5$GAGG(N)$_7$▲…5'	2	4	2	4	4
BsrDI	Cyt *b*	5'…GCAATGNN▼…3' 3'…CGTTAC▲NN…5'	1	0	0	0	0

5.5.2 Recognition sites of the selected enzymes

Using the virtual digest, the recognition sites of the enzymes could be identified to calculate fragment length, see table 5.5 for the expected fragment lengths and tables 5.6, 5.7 and 5.8 for the recognition sites in Cracid species.

Table 5.5: Restriction fragment length in *Pipile pipile* and *Pipile cumanensis grayi* when digested with *EcoRV*, *BsaXI* and *BsrDI*.

Species	*EcoRV* (size in bp)	*BsaXI* (size in bp)	*BsrDI* (size in bp)
Pipile pipile	Undigested	30 201 377	551 245
Pipile cumanensis grayi	157 454	30 (x2) 119 201 228	Undigested

Table 5.6: Recognition site for *EcoRV* in ND2

Species	G	A	T	A	T	C
P.unicornis	.	.	.	.	C	A
P.pauxi	.	.	.	.	C	A
C. blumenbachii	.	G	.	.	C	A
C.rubra	.	.	.	.	C	A
C.globulosa	.	G	.	.	C	A
C.fasciolata	.	.	.	.	C	A
C.daubentoni	.	.	.	.	C	A
C.alector	.	.	.	.	C	A
C.alberti	.	.	.	.	C	A
N.urumutum	.	.	.	.	C	A
M.tomentosa	.	.	.	.	C	A
M.salvini	.	.	.	.	C	A
M.mitu	.	.	.	.	C	A
M.tuberosa	.	.	.	.	C	A
O.derbianus	.	.	.	.	C	A
O.canicollis	.	.	.	.	C	A
P.nigra	.	.	.	.	C	A
C.goudotii	.	.	.	.	C	A
A.aburria	.	G	.	.	.	.
P.jacutinga	.	G	.	.	.	.
P.pipile	.	G	.	.	.	.
P. cumanensis	.	.	.	.	.	.
P. cumanensis grayi	.	.	.	.	.	.
P.cujubi	.		.	.	.	

Table 5.7: First recognition site of *BsaXI* within ND2

Species	A	C	$(N)_5$	C	T	C	C
P.unicornis	.	.	/	.	C	.	.
P.pauxi	.	.	/	.	C	.	.
C. blumenbachii	.	.	/	.	C	.	.
C.rubra	.	.	/	.	C	.	.
C.globulosa	.	.	/	.	C	.	.
C.fasciolata	.	.	/	.	C	.	.
C.daubentoni	.	.	/	.	C	.	.
C.alector	.	.	/	.	C	.	.
C.alberti	.	.	/	.	C	.	.
N.urumutum	.	.	/	.	C	.	.
M.tomentosa	.	.	/	.	.	.	.
M.salvini	.	.	/	.	.	.	.
M.mitu	.	.	/	.	.	.	.
M.tuberosa	.	.	/	.	.	.	.
O.derbianus	.	.	/	.	C	.	.
O.canicollis	.	.	/	.	C	.	.
P.nigra	.	.	/	.	C	.	.
C.goudotii	.	.	/	.	C	.	.
A.aburria	.	.	/	.	.	.	.
P.jacutinga	.	.	/	.	.	.	.
P.pipile	.	.	/	.	.	.	.
P. cumanensis	.	.	/	.	.	.	.
P. cumanensis grayi	.	.	/	.	.	.	.
P.cujubi	.	.	/	.	.	.	.

Table 5.8: Second recognition of *BsaXI* within ND2

Species	A	C	$(N)_5$	C	T	C	C
P.unicornis	.	.	/	.	.	T	.
P.pauxi	.	.	/	.	.	T	.
C. blumenbachii	.	.	/	.	.	T	.
C.rubra	.	.	/	.	.	T	.
C.globulosa	.	.	/	.	.	T	.
C.fasciolata	.	.	/	.	.	T	.
C.daubentoni	.	.	/	.	.	T	.
C.alector	.	.	/	.	.	T	.
C.alberti	.	.	/	.	.	T	.
N.urumutum	.	.	/	.	.	T	.
M.tomentosa	.	.	/	.	.	T	.
M.salvini	.	.	/	.	.	T	.
M.mitu	.	.	/	.	.	T	.
M.tuberosa	.	.	/	.	.	T	.
O.derbianus	.	T	/	.	.	T	.
O.canicollis	.	.	/	.	.	T	.
P.nigra	.	.	/	.	.	T	.
C.goudotii	.	T	/	.	.	.	.
A.aburria	G	.	/	.	.	.	.
P.jacutinga	G	.	/	.	.	.	.
P.pipile	G	.	/	.	.	.	.
P. cumanensis	.	.	/	.	.	.	.
P. cumanensis grayi	.	.	/	.	.	.	.
P.cujubi	.	.	/	.	.	.	.

Restriction polymorphisms successfully distinguish *Pipile pipile* from *Pipile cumanensis* and *Pipile cumanensis grayi* however the alignments of the recognition sites show that an unknown sample could be assigned to *Pipile pipile*, *Pipile jacutinga*, *Aburria aburri* or any of the 4 *Mitu* species listed. Although distinction would not be required in Trinidad, separating *Pipile pipile* from other Cracid species provides confidence that the method will allow separation in more distantly related species. Therefore to further distinguish species identification and overcome the possibility of mutation at the restriction site, *BsrDI* was used to provide further distinction. By analysing the cyt *b* sequencing and identifying the recognition site, as seen in table 5.9, it can be inferred that this restriction enzyme could distinguish *Pipile pipile* from all other Cracids (for which data is currently available) except *Chamaepetes goudotii*. However, if used in conjunction with restriction enzymes of the ND2 digest, this species can be excluded.

Table 5.9: Recognition site for *BsrDI* in cyt *b*

Species	G	C	A	A	T	G
P.unicornis	.	.	.	.	.	A
P.pauxi	.	.	.	.	.	A
C.pauxi	.	.	.	.	.	A
C.rubra	.	.	.	.	.	A
C.globulosa	.	.	.	.	.	A
C.fasciolata	.	.	.	.	.	A
C.daubentoni	.	.	.	.	.	A
C.alector	.	.	.	.	.	A
N.urumutum	.	.	.	.	.	A
M.tomentosa	.	.	.	.	.	A
M.salvini	.	.	.	.	.	A
M.mitu	.	.	.	.	.	A
M.tuberosa	.	.	.	.	.	A
O.derbianus	A	.	.	.	.	A
O.canicollis	A	.	.	.	.	A
P.nigra	.	.	.	.	.	A
C.goudotii	.	.	.	.	.	.
A.aburria	.	.	.	.	.	A
P.jacutinga	.	.	.	.	.	A
P.pipile	.	.	.	.	.	.
P. cumanensis	.	.	.	.	.	A
P. cumanensis grayi	.	.	.	.	.	A
P.cujubi	.	.	.	.	.	A

5.6 Analysis of field samples using species detection methods

Methodologies established using data from the reference specimen of the Trinidad Piping guan were tested on field samples and captive birds to substantiate the techniques. All samples underwent universal DNA amplification to avoid false negatives followed by the designed species specific primer and RFLP methods. In addition, samples were sequenced following experimentation to confirm species origin. Three samples were received from the University of West Indies which had been collected within Trinidad Piping guan habitat over a period of two years. The small collection size obtained during this time demonstrates the difficulties in retrieving samples due to the elusiveness of the species.

5.6.1 Moulted feather quill sample

The first of the Trinidad field samples originated from a flight feather found on the forest floor of Morne Bleu shortly after a bird had flown from the area. Researchers could not identify the species but the size and colour of the feather was characteristic of the Trinidad Piping guan. The quill tip of this feather was received for genetic analysis, which on receipt was stored at -17^{o}C (figure 5.6).

Fig. 5.6: Quill tip received from the University of West Indies taken from a moulted flight feather found on the forest floor of Morne Bleu.

5.6.2 Fallen birds egg

The second sample which was received from the University of West Indies originated from an egg which had fallen from a nest. The egg was found at the foot of a tree in which the landowner believed Trinidad Piping guan to be nesting. Finding the egg cracked and cold the discovery was reported to the Pawi Study Group who acquired the egg and also the abandoned nest materials for further analysis.

After external examination of the egg, an X-ray was performed to assess any possible contents. On discovery of fertilisation, the decision was taken to open the egg for examination. Although origin was unconfirmed, this was treated as the first study of a Trinidad Piping guan egg. When opened, an embryo in the late stages of development was found with characteristics pertaining to the Trinidad Piping guan (see figure 5.7a-d for images).

Fig. 5.7a-d: a) The egg received from the Pawi Study Group with a Trinidad 10cent coin (1.63cm in diameter) for reference. b) Radiograph of the egg. c) Ventral aspect of embryo. d) Dorsal aspect of embryo.
Photographs provided by Prof. John Cooper

Because the morphology of embryonic *Pipile pipile* is un-described and feathers are developing and cannot therefore undergo barbule analysis, species classification could not be confirmed. It was therefore asked that the methods of identification developed within this study be used to identify the embryo to establish species origin. Samples were taken from the liver of the embryo, developing feathers and eggshell containing the chorioallantoic membrane. When received, these samples were stored overnight at 4^{o}C before DNA extraction was performed the following day; all remaining samples were then stored at -17^{o}C. It is possible to extract maternal DNA from the outer eggshell (Schmaltz, Somers, Sharma & Quinn, 2006; Egloff, Labrosse, Hebert & Crump, 2009) however the external surface had been washed prior to export and this extraction was therefore not viable. In addition, as the analysis focuses on mtDNA, the genetic data would be identical due to maternal inheritance and therefore unnecessary. Though these sample types could be classed as invasive, the manor in which they were obtained moves the classification to non-invasive as their collection would not have had an adverse effect on the population. With respect to the samples of eggshell, these could be obtained non-invasively by collection from the nests of recently hatched chicks. Care must be taken when obtaining this sample not to disturb the nest or provide any reason for it to be abandoned by the parents. Obtaining this sample type in future studies of the species would however prove difficult due to the location of nests within the tree canopy.

5.6.3 Second moulted feather sample

The third sample received was again a feather sample, which due to its smaller size was received intact (see figure 5.8). This feather was collected in known Trinidad Piping guan habitat near the Plate Plantation house in Grande Riviere. From visual analysis, the feather was suspected to originate from the wing due to the presence of vanes and although much smaller than previous flight feathers received, it was possible that this was a primary or secondary covert.

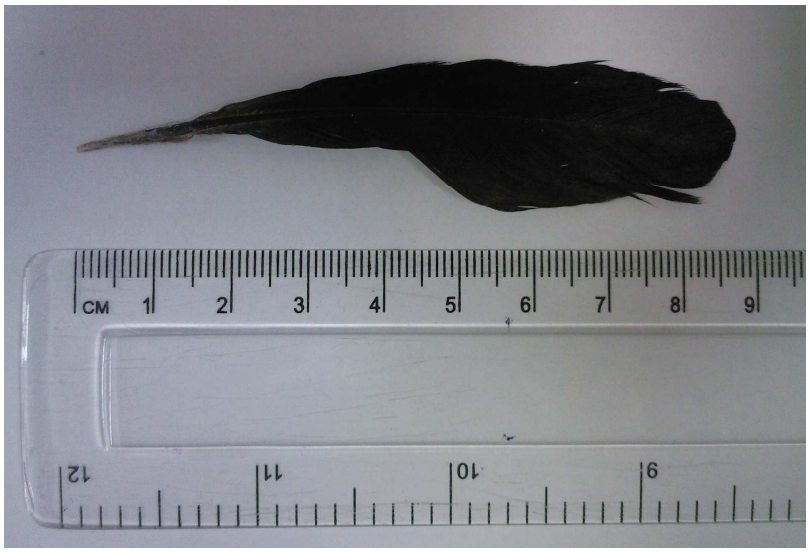


Fig. 5.8: Feather received from the Grande Riviere, Trinidad

5.7 UK population of Piping guan

According to the International Species Identification System (ISIS) database (data obtained September 17, 2009), a UK breeding population of the Trinidad Piping guan exists with a male and female held at the Cotswold Wildlife Park and Gardens in Oxfordshire and a male and two females at the Lotherton Bird Garden in Leeds (International Species Identification System, n.d.). One of the birds held at the Cotswold Wildlife Park and Gardens can be seen in figure 5.9.

The parks were contacted to find out more information regarding the origin of these individuals as there is no record of any bird being removed from the island since the species became legally protected in 1958. During conversations with both bird handlers it was discovered that the true species identification was unknown and the birds had been donated by another collector. Discussions were held with this original collector from Fenton Barns in East Lothian who claimed that the true identification of the species was not known and that the current assessment had been performed by another collector's morphological analysis (K. Chalmers-Watson, personal communication, November 8, 2009). Two more birds were held by the collector at Fenton Barns which were not yet registered on the ISIS database.

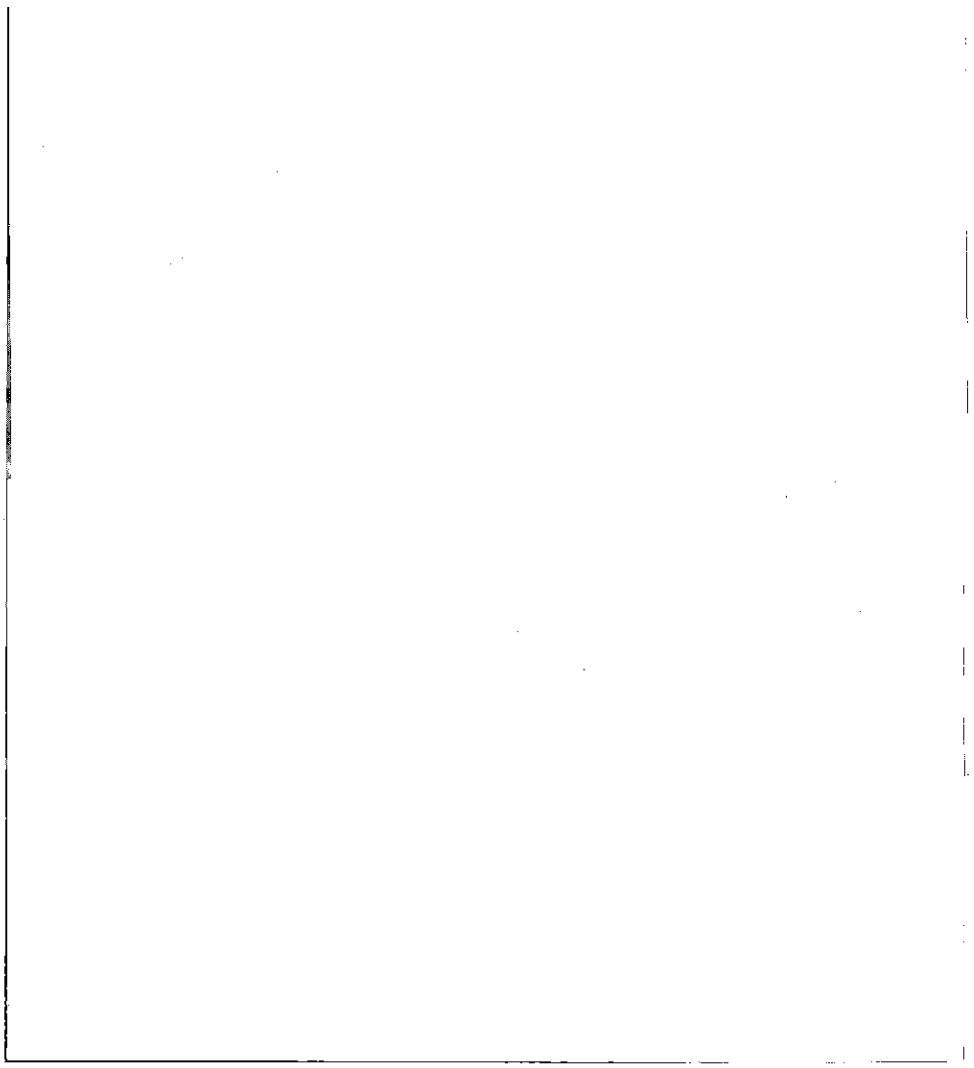


Fig. 5.9: Bird held at the Cotswold Wildlife Park and Gardens, this bird displays morphological characteristics similar to the Trinidad Piping guan, most strikingly the blue dewlap.
Photograph provided by Louise Peat

5.7.1 Samples obtained from UK Piping guans

To attempt genetic classification of these birds, all three handlers subsequently agreed to donate feather samples for identification purposes. Though moulted feather samples were requested for analysis, Fenton Barns routinely pluck breast feathers for DNA sexing. As these handlers are experienced with feather removal, two plucked feathers from each of the birds were accepted for analysis. Lotherton and Burford collected moulted feather samples from all birds over a

number of months. When received, all samples were stored at -17^{o}C. It was found that the Lotherton Bird Garden were running a successful breeding programme and four more birds had been born since registering with ISIS. By speaking with the handlers about the parentage of each bird it was possible to trace the mitochondrial lineages in order to establish which birds to focus our analysis. Figure 5.10 shows the genetic line of the UK population of Piping guans. From this lineage assessment we can identify that the two breeding pairs at Fenton Barns and Lotherton Bird Garden must undergo analysis, the results of which can be extrapolated for the Lotherton offspring and to inform Burford of the species assignment.

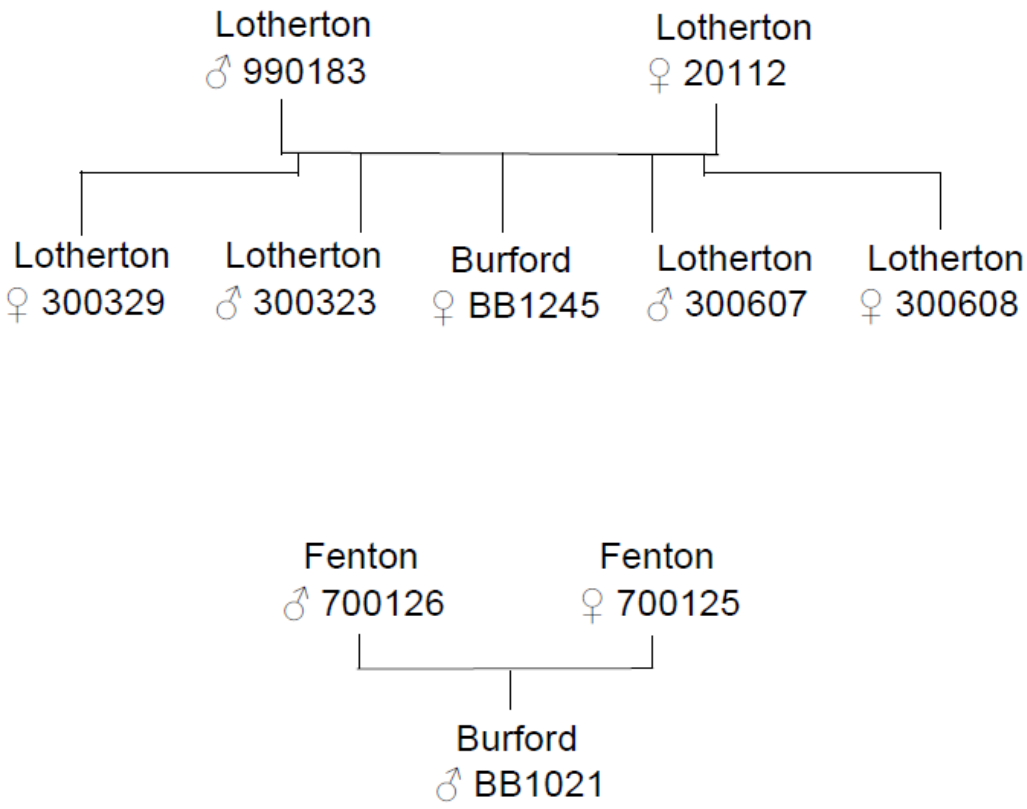


Fig. 5.10: Genetic lineage of the UK population of Pipile pipile*, the bird park name and individual identification number are used to classify each individual*

5.8 DNA extraction

DNA was extracted from the Trinidad and UK samples using Qiagen DNeasy blood and tissue kit as described in chapter 2. DNA was extracted from 5-10mm of the inferior umbilicus of all feather samples and for continuity of sample type; DNA extracted from the developing feathers of the embryo to perform the following analyses.

5.8.1 Species specific primer amplification for ND2 and cyt **b**

All PCR reactions were set up as described in chapter 2 and visualised on a 2% agarose gel pre-stained with Biotium GelRed™. Gels were exposed under UV light and contained a 100bp ladder PCRsizer as reference. ND2 species primers LPawi1 (5'-GCAGCCCTAATACTCACCTTACTT-3') and HPawi1 (5'-AAGAATAGGCCCAGGAGTGATAGC-3') were amplified using PCR conditions of an initial denaturation of 5mins at 94^{o}C followed by 30 cycles of 1 min at 94^{o}C, 1min at 65^{o}C and 1 min at 72^{o}C with a final extension of 10mins at 72^{o}C. The cyt *b* species specific primers LPawi2 (5'-AAACGTCCAATACGGCTGG-3') and HPawi2 (5'-GCTCGTTGTTTGGATTTGTGTAGA-3') underwent PCR of 5mins at 94^{o}C followed by 30 cycles of 1min at 94^{o}C, 1min at 60^{o}C and 1min at 72^{o}C with a final extension of 10mins at 72^{o}C.

5.8.2 Amplification of ND2 and cyt **b** *fragments for RFLP analysis*

The second half of the ND2 gene was amplified for RFLP analysis using L5758 (5'-GGNGGNTGAATRGGNYTNAAYCARAC-3') and H6313 (5'-ACTCTTRTTT AAGGCTTTGAAGGC-3') previously designed by Sorenson (2003). PCR conditions consisted of an initial denaturation of 5mins at 94^{o}C followed by 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 2mins at 72^{o}C with a final extension of 30mins at 72^{o}C.

DNA amplification for the purpose of cyt *b* restriction digest was amplified using primers L15311 (5'-CTCCCATGAGGCCAAATATC-3') and H16065 (5'-TTCAGTTTTTGGTTTACAAGAC-3') (Kimball, Braun, Zwartjes, Crowe & Ligon,

1999). Reaction conditions were 5min at 94^{o}C and 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 1min at 72^{o}C and a final extension of 5min at 72^{o}C.

Restriction enzymes were obtained from New England Biolabs, using the recommended 1x NEBuffer 4 for BsaXI, 1x NEBuffer 3 for *EcoRV* and 1x NEBuffer 2 for *BsrDI*. Amplifications were digested in 4000units of each restriction enzyme performed in separate 50µl reaction volumes. ND2 restriction digests were incubated at 37^{o}C overnight and the cyt *b* restriction digest was incubated at 60^{o}C overnight to produce 100% activity. Fragment patterns were observed on a 2% agarose gel visualised using Biotium GelRed™ under exposure with UV light using 100bp ladder PCRsizer as reference. As both ND2 enzymes have the same reaction temperature it is possible to conduct a double digest using NEbuffer 2 which has an activity of 100% in *BsaXI* and 75% in *EcoRV*. However, this double digest was not conducted in order to provide clearer results and to maintain the 100% reactivity in *EcoRV*.

5.8.3 Sequencing confirmation

To improve the confidence of the species detection tests sequencing was performed for comparative analysis. The first half of the ND2 gene and a fragment of cyt *b* were also amplified to confirm species origin of the UK Piping guans. Therefore, primers L5216 (5'-GGCCCATACCCCGRAAATG-3') and H5766 (5'-RGAKGAGAARGCYAGGATYTTKCG-3') were used to amplify the first half of ND2 with primer conditions of 5mins at 94^{o}C followed by 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 2mins at 72^{o}C with a final extension of 30mins at 72^{o}C. A further cyt *b* fragment was amplified using L14990 (5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3') and H15696 (5'-AATAGGAAGTATCATTCGGGTTTGATG-3') with PCR conditions of initial denaturation at 94^{o}C for 5mins followed by 40 cycles of 1min at 94^{o}C, 45s at 50^{o}C and 2min at 72^{o}C with a final extension of 10mins at 72^{o}C (Kornegay, Kocher, Williams & Wilson, 1993) for identification.

5.8.4 Universal primers

In order to examine all DNA samples for the presence of DNA to avoid the possibility of a false negative with the developed species detection tests, universal primers were used to amplify a small fragment of DNA. Primers were that designed by Kocher et al., (1989) consisting of L14841 (5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3') and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'). PCR conditions were developed from protocol in Kocher et al. (1989) with an initial denaturation of 94^{o}C for 5mins and 40 cycles of 1min at 94^{o}C, 1min at 55^{o}C and 2mins at 72^{o}C with a final extension of 30mins at 72^{o}C.

5.8.5 Sanger sequencing and manipulation of data

All sequencing was performed by MWG Eurofins using the amplification primers. DNA was quantified and purified by MWG and results downloaded from the MWG Eurofins website. Forward and reverse sequencing was compared using FASTA Sequence Comparison (Pearson, 2006) and only matching double stranded sequencing data with a phred score of ≥30 (99.9% accuracy) was used for further analysis. Sequences were aligned using ClustalX (Thompson et al., 1997) and when converted to postscript viewed using GSview 4.9 (Lang, 2007). All oligonucleotide sequences of designed species specific primers can be seen in table 5.10 which also lists the amplification/sequencing primers used previously to obtain sequence data from *Pipile piple* and *Pipile cumanensis grayi* and universal primers to test for the presence of DNA.

Table 5.10: All primers used within this chapter displaying the study from which they were derived.

Primer	Gene	Sequence	Taken from
LPawi1	ND2	5'-GCAGCCCTAATACTCACCTTACTT-3'	Current study
HPawi1	ND2	5'-AAGAATAGGCCCAGGAGTGATAGC-3'	Current study
LPawi2	Cyt *b*	5'-AAACGTCCAATACGGCTGG-3'	Current study
HPawi2	Cyt *b*	5'- CTACTAGGGTTTGTCCG-3'	Current study
L5216	ND2	5'-GGCCCATACCCCGRAAATG-3'	Sorenson (2003)
H5766	ND2	5'-RGAKGAGAARGCYAGGATYTTKCG-3'	Sorenson (2003)
L5758	ND2	5'-GGNGGNTGAATRGGN YTNAAYCARAC-3'	Sorenson (2003)
H6313	ND2	5'-ACTCTTRTTTAAGGCTTTGAAGGC-3'	Sorenson (2003)
L15311	Cyt *b*	5'-CTCCCATGAGGCCAAATATC-3'	Kimball et al. (1999)
H16065	Cyt *b*	5'-TTCAGTTTTTGGTTTACAAGA-3'	Kimball et al. (1999)
L14990	Cyt *b*	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'	Kornegay et al. (1993)
H15696	Cyt *b*	5'-AATAGGAAGTATCATTCGGGTTTGATG-3'	Kornegay et al. (1993)
L14841	Cyt *b*	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'	Kocher et al. (1989)
H15149	Cyt *b*	5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'	Kocher et al. (1989)

Results

5.9 Initial test of DNA presence with universal primers

To ensure the presence of genetic material in each of the DNA extractions, all samples were amplified using universal primers. The results of this preliminary test can be seen in figure 5.11 and corresponding table 5.11. This gel identifies the presence of DNA in all extractions.

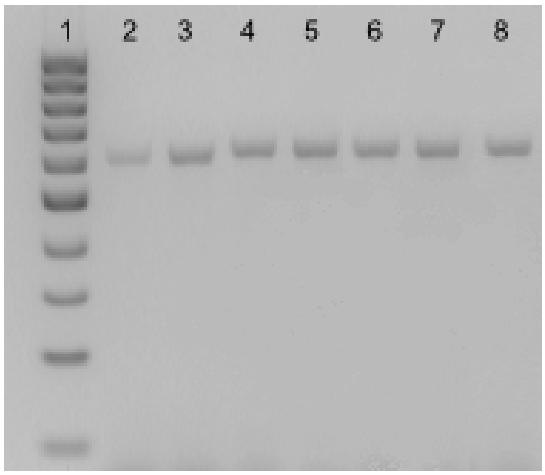


Fig. 5.11: Initial test to identify the presence of DNA in the original extractions

Table 5.11: All DNA extracts amplified with universal primers

Lane	Primer Set	Sample
1	N/A	100bp ladder
2	L14841/H15149	Unknown quill
3	L14841/H15149	Embryo
4	L14841/H15149	Unknown feather
5	L14841/H15149	Fenton male
6	L14841/H15149	Fenton female
7	L14841/H15149	Lotherton male
8	L14841/H15149	Lotherton female

5.9.1 Analysis of the ND2 species specific primers

The species specific primers for *Pipile piple* originally designed within the ND2 gene were tested not only on *Pipile* species but also on other closely related species (*Crax blumenbachii* and *Crax fasciolata*), common domestic fowl (*Gallus gallus domesticus*) and morphologically similar species (*Coragyps atratus*). The results for the ND2 primer set can be seen in figure 5.12 and table 5.12.

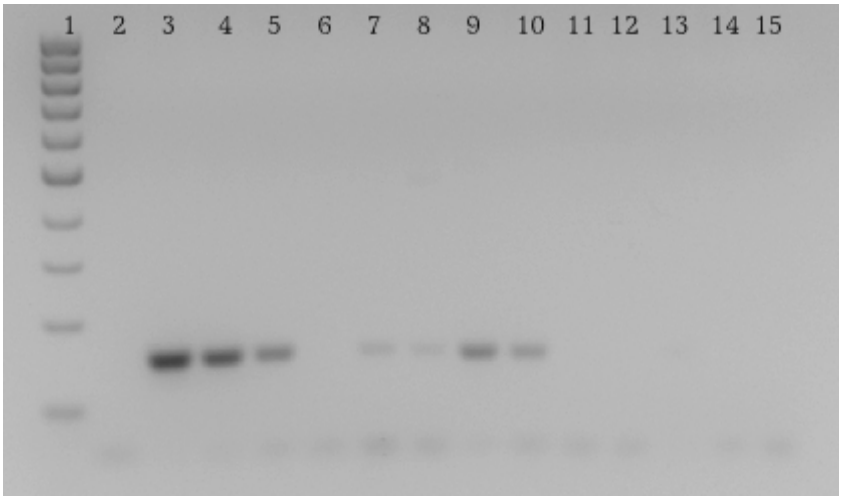


Fig. 5.12: 2% agarose gel electrophoresis of the ND2 species specific primer amplification on multiple species. Descriptions of lanes 3-15 are given in table 5.12.

Table 5.12: Species tested by PCR for the validity of the ND2 species primers

Lane	Primer Set	Sample	Common Name
1	N/A	100bp ladder	N/A
2	Pawi1	Negative control	N/A
3	Pawi1	*Pipile pipile*	Trinidad Piping guan
4	Pawi1	Unknown quill	Unknown
5	Pawi1	Embryo	Unknown
6	Pawi1	Unknown feather	Unknown
7	Pawi1	Fenton male	Unknown
8	Pawi1	Fenton female	Unknown
9	Pawi1	Lotherton male	Unknown
10	Pawi1	Lotherton female	Unknown
11	Pawi1	*Pipile cumanensis grayi*	Gray's Piping guan
12	Pawi1	*Coragyps atratus*	Black Vulture
13	Pawi1	*Crax blumenbachii*	Red-billed Curassow
14	Pawi1	*Crax fasciolata*	Bare-faced Curassow
15	Pawi1	*Gallus gallus domesticus*	Domestic Chicken

A strong amplification can be seen in lanes 3-5 which contain the reference *Pipile pipile* and field samples from the feather quill and egg. The third sample received from Trinidad of the unknown feather did not exhibit amplification, providing evidence that it originated from a different species. Lanes 7-10 containing the UK Piping guans are seen to also produce amplification but of a lower density than the Trinidad samples. On this gel it can be seen that the voucher sample of *Pipile cumanensis grayi* does not produce amplification.

5.9.2 Analysis of the cyt b *species specific primers*

The same samples were examined with the cyt *b* species specific primers; the results of this analysis can be seen in figure 5.13 and table 5.13.

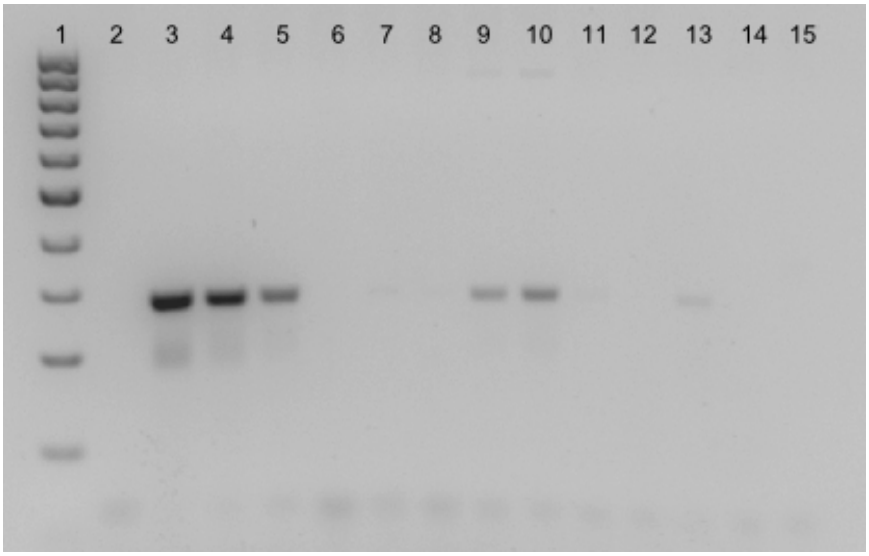


Fig. 5.13: Agarose gel electrophoresis of the cyt b species primer tested on multiple species. Descriptions of lanes 3-15 are given in table 5.13.

Table 5.13: Species tested by PCR for the validity of the cyt *b* species primers

Lane	Primer Set	Sample	Common Name
1	N/A	100bp ladder	N/A
2	Pawi2	Negative control	N/A
3	Pawi2	*Pipile pipile*	Trinidad Piping guan
4	Pawi2	Unknown quill	Unknown
5	Pawi2	Embryo	Unknown
6	Pawi2	Unknown feather	Unknown
7	Pawi2	Fenton male	Unknown
8	Pawi2	Fenton female	Unknown
9	Pawi2	Lotherton male	Unknown
10	Pawi2	Lotherton female	Unknown
11	Pawi2	*Pipile cumanensis grayi*	Gray's Piping guan
12	Pawi2	*Coragyps atratus*	Black Vulture
13	Pawi2	*Crax blumenbachii*	Red-billed Curassow
14	Pawi2	*Crax fasciolata*	Bare-faced Curassow
15	Pawi2	*Gallus gallus domesticus*	Domestic Chicken

The cyt *b* primers also produce a strong amplification for 2 of the 3 Trinidad samples received during the course of the study. Amplification can also be seen in lanes 9 and 10 and very faintly in lanes 7, 8 and 11. A small amplification can also be seen in lane 13 for *Crax blumenbachii*. At this point all unclassified samples except the unknown Trinidad feather warrant further investigation by RFLP analysis as all produce amplification with the species specific primers. This initial test of species detection therefore allows samples to be excluded from analysis, minimising costs of an investigation.

5.9.3 RFLP analysis of ND2 digest

A single restriction digest was performed for each chosen enzyme in order to produce 100% enzyme activity. The results from digestion with *EcoRV* can be seen in figure 5.14 and table 5.14.

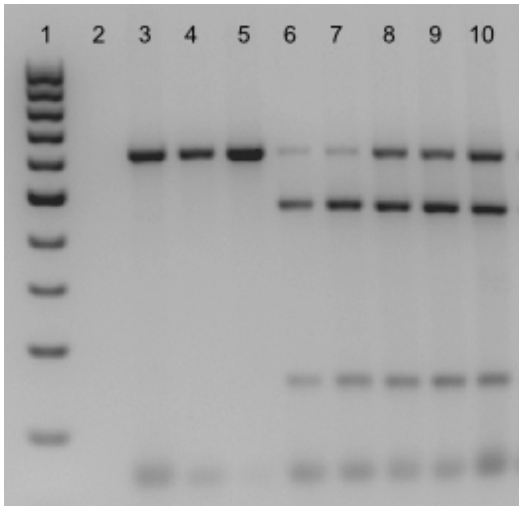


Fig. 5.14: 2% agarose gel electrophoresis of the restriction digest of Trinidad and UK samples with EcoRV. Descriptions of lanes 1-10 are given in table 5.14.

Table 5.14: Samples digested with *EcoRV*

Lane	Enzyme	Sample	Common Name
1	N/A	100bp Ladder	N/A
2	*EcoRV*	Negative control	N/A
3	*EcoRV*	*Pipile pipile*	Trinidad Piping guan
4	*EcoRV*	Unknown quill	Unknown
5	*EcoRV*	Embryo	Unknown
6	*EcoRV*	Fenton male	Unknown
7	*EcoRV*	Fenton female	Unknown
8	*EcoRV*	Lotherton male	Unknown
9	*EcoRV*	Lotherton female	Unknown
10	*EcoRV*	*Pipile cumanensis grayi*	Gray's Piping guan

The Trinidad samples are seen to remain undigested with *EcoRV* with all UK Piping guans and the reference *Pipile cumanensis grayi* undergoing a single digest producing the expected fragment lengths of 157 and 454bp. Some amplification is seen to remain undigested due to the quantity of DNA present in the reaction. This provides evidence for the Trinidad samples originating from

Pipile pipile and the UK birds from *Pipile cumanensis grayi*. These samples were then digested with *BsaXI*, the results of which can be seen in figure 5.15 and table 5.15.

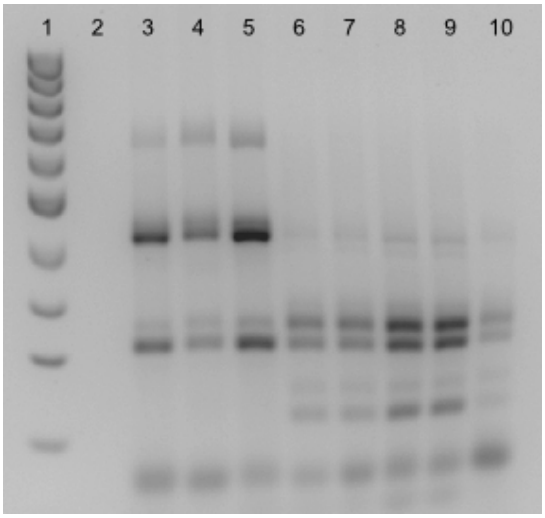


Fig. 5.15: 2% agarose gel electrophoresis of the restriction digest of Trinidad and UK samples with BsaXI. Descriptions of lanes 1-10 are given in table 5.15.

Table 5.15: Samples digested with *BsaXI*

Lane	Enzyme	Sample	Common Name
1	N/A	100bp Ladder	N/A
2	*BsaXI*	Negative control	N/A
3	*BsaXI*	*Pipile pipile*	Trinidad Piping guan
4	*BsaXI*	Unknown quill	Unknown
5	*BsaXI*	Embryo	Unknown
6	*BsaXI*	Fenton male	Unknown
7	*BsaXI*	Fenton female	Unknown
8	*BsaXI*	Lotherton male	Unknown
9	*BsaXI*	Lotherton female	Unknown
10	*BsaXI*	*Pipile cumanensis grayi*	Gray's Piping guan

A clear distinction can again be seen between samples originating from Trinidad and those from the UK. The most distinctive band in this restriction digest is the fragment length of 119bp. This fragment occurs between the two restriction sites in *Pipile cumanensis grayi* and because this second site does not occur in *Pipile pipile*, it is species specific. Therefore, if this band is present species origin can be confirmed even if some unexpected banding occurs.

5.9.4 RFLP analysis of the cyt b *digest*

Only one enzyme *(BsrDI)* in the electronic digest was seen to be successful in distinguishing *Pipile pipile* from *Pipile cumanensis grayi* when using cyt *b.* The banding pattern produced after digestion with this enzyme can be seen in figure 5.16 with descriptions in table 5.16. This reaction produces a very distinctive result which easily distinguishes *Pipile pipile* from *Pipile cumanensis grayi*.

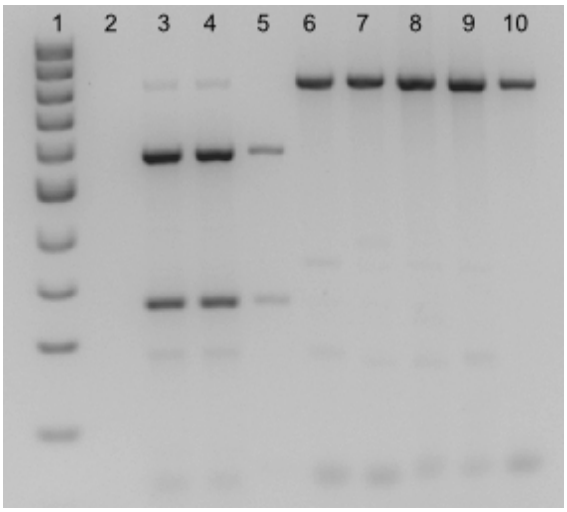


Fig. 5.16: 2% agarose gel electrophoresis of the restriction digest of Trinidad and UK samples with BsrDI. Descriptions of lanes 1-10 are given in table 5.16.

Table 5.16: Samples digested with *BsrDI*

Lane	Enzyme	Sample	Common Name
1	N/A	100bp Ladder	N/A
2	*BsrDI*	Negative control	N/A
3	*BsrDI*	*Pipile pipile*	Trinidad Piping guan
4	*BsrDI*	Unknown quill	Unknown
5	*BsrDI*	Embryo	Unknown
6	*BsrDI*	Fenton male	Unknown
7	*BsrDI*	Fenton female	Unknown
8	*BsrDI*	Lotherton male	Unknown
9	*BsrDI*	Lotherton female	Unknown
10	*BsrDI*	*Pipile cumanensis grayi*	Gray's Piping guan

5.10 Sequencing confirmation

In order to verify the results of the previous species detection tests, the original ND2 amplification of the RFLP analysis was sequenced to provide species identification. For further confirmation of results, the first half of the ND2 gene was also amplified as this region has been shown to contain a higher level of variation. It was decided that sequencing of cyt *b* should be conducted on a different region of the gene for the UK samples than that which was amplified for use in RFLP analysis as previous sequencing alignments have shown very little variation to occur. To improve genetic data available for the Trinidad Piping guan, full cyt *b* gene sequencing was performed on all Trinidad samples. Although samples need only to be excluded in a species detection test, the cyt *b* fragment containing variation was amplified in order to identify the UK samples of Piping guan. This volume of sequencing would not be required in a case looking to simply confirm the identity of a sample which tested positive for all *Pipile pipile* detection tests. The third Trinidad sample (the unknown feather) was sequenced from the initial DNA quantity test using the universal primer pair in order to confirm species origin was not Trinidad Piping guan and further verify the methodologies created for species detection.

5.10.1 ND2 sequence alignment

Using the SNPs seen in the *Pipile pipile* sequence data identified in chapter 4, the UK Piping guans and Trinidad samples were compared to the reference data of *Pipile pipile*. It was found that the Trinidad samples exhibited a 100% match with the reference data and species was therefore classed as *Pipile pipile*. The SNPs available in the ND2 gene did not match *Pipile pipile* SNPs for any of the UK Piping guan samples. This therefore provided evidence, as previously suspected, that these birds are not *Pipile pipile*.

5.10.2 Cyt b *sequence alignment*

When the quill sample was compared to the reference *Pipile pipile* data, the sequences were seen to match 100% thereby confirming species identification as *Pipile pipile*. However, when comparing the embryo sequence data, a tenth SNP was identified at position 1004 matching neither the reference *Pipile pipile* nor voucher *Pipile cumanensis grayi*. This SNP was observed to have a high phred score and was accepted as intra-species variability. All other previously observed SNP positions matched *Pipile pipile* and therefore species assignment was confirmed as *Pipile pipile*. When the UK Piping guan samples were compared to these same SNPs, none matched *Pipile pipile* at any base, it can therefore be confirmed that these samples are not Trinidad Piping guan.

5.10.3 Identification of the field feather

Sequencing obtained using the universal amplification primers on the unknown flight feather was subjected to a BLAST search on the NCBI website. This search of the nucleotide database indicated a high similarity score with gull species (*Larus*) and of the 100 similarity matches, none of Cracid species were returned. A 100% match was not obtained by this search, most likely due to the species of origin being a genetically unstudied island bird. Together these results demonstrate the reliability of the species detection methods to eliminate non-target species at an early stage of investigation.

Discussion

5.11 Success of the species specific methods

The limited performance of the species specific primers is the result of low inter-species genetic variability. With few SNPs available for inclusion within the primers, the specificity decreases enabling closely related species to be amplified. Though not an issue in field or forensic studies where no other Cracid is found in Trinidad, classification of captive birds requires RFLP analysis.

The restriction enzymes enable classification of species with the use of multiple genes and sites providing strong support for identification. Positive results achieved by this analysis in field studies can therefore confirm identity of derivatives originating from *Pipile pipile*. If positive results were seen in a case of forensic investigation, such as identifying wildmeats, sequence analysis may then be required for use as evidence of species identification.

5.12 Sequence similarity

The minimal amount of sequence variation found between *Pipile pipile* samples can in some way be explained by the species low population numbers. Although the present study only incorporates 3 individuals of the species, if estimations are correct and only 200 birds remain this represents 1.5% of the entire population. As population numbers decrease and therefore variant alleles are removed, the reduction of gene flow reduces the number of mitochondrial haplotypes. In some cases, mtDNA can then become species-specific in a reduced population not constrained by geographical boundaries. Due to the maternal inheritance of mtDNA and its reduced gene number, a population can be seen to descend from a single female lineage within $4N$ (where N is the population size) generations (Cronin et al., 1991).

The low intra-species genetic diversity evident in the current study may therefore be explained not necessarily in terms of a low level of haplotypes from a founder population but possibly as a result of genetic bottlenecking. Over time the effects of hunting and habitat loss may have engendered a decrease in gene flow resulting in an overall loss of genetic variation within the population. The reduction in species numbers may have therefore created a panmictic population within a small habitat region, possibly creating an issue of inbreeding.

5.12.1 Comparing **Pipile cumanensis** *and* **Pipile cumanensis grayi**

By comparing the full gene data for *Pipile cumanensis grayi* with sequence data deposited on GenBank for *Pipile cumanensis*, four SNPs were located within ND2 and two SNPs within cyt *b* which distinguish *Pipile cumanensis* from *Pipile*

cumanensis grayi (see appendices VI and VII) However, these SNPs do not appear to be species defining as only one UK bird (Lotherton male) matched 100% to *Pipile cumanensis grayi* and the remaining 3 did not match 100% to either *Pipile cumanensis* or *Pipile cumanensis grayi*. Of the 6 SNPs each of the remaining birds contained some mutations seen only in *Pipile cumanensis* with the Fenton male and Lotherton female containing 1 of 6 and the Fenton female containing 4 of 6. Of these SNPs, 5 occurred at the third base position and only one appeared at a first base position, this SNP is seen in 3 of the UK birds (see table 5.17). Although SNPs which alter the amino acid sequence, in this case from phenylanine in *Pipile cumanensis* to leucine in *Pipile cumanensis grayi,* are normally more informative for species distinction, the majority of SNPs corroborate with sequencing from *Pipile cumanensis grayi*. Also, though a change in amino acid is seen, both of these are non-polar, hydrophobic amino acids which therefore may not have an adverse effect on the protein (a neutral mutation).

Table 5.17: Nucleotide expressed at each of the 6 SNPs seen between *Pipile cumanensis* and *Pipile cumanensis grayi* within cyt *b* and ND2. Those which match the GenBank data of *Pipile cumanensis* are shaded; all other bases match *Pipile cumanensis grayi.*

UK Piping guans	cyt *b*		ND2			
	1	2	3	4	5	6
Fenton male	T	T	T	A	G	G
Fenton female	T	T	T	G	A	A
Lotherton male	T	C	T	A	G	G
Lotherton female	T	T	T	A	G	G

Although distinction between these sub-species would not be required for detection of *Pipile pipile*, it is worth noting the significant similarity in the gene sequences of *Pipile cumanensis* and *Pipile cumanensis grayi* which may require further scrutiny in future investigations. This is demonstrated by the Fenton female bird of which 4 of 6 bases correspond to *Pipile cumanensis*. It was assumed due to the birds previous classification of *Pipile pipile* that all had the

distinctive blue dewlap which defines the species. However, it is possible that the Fenton female should actually be classified as *Pipile cumanensis* (which has a dark blue/purple dewlap) rather that *Pipile cumanensis grayi* with the offspring of the Fenton breeding pair (the Burford male) being a hybrid. Should distinction of these species ever be required when morphology cannot be assessed, sequencing regions of the D-loop may be necessary in order to define the species by mtDNA.

In the current investigation, morphological evidence is available to assist species identification and would be available in all studies of captive birds. Due to the darker dewlap colour of *Pipile cumanensis* the bird seen in figure 5.9 can be classified as *Pipile cumanensis grayi* using genetic techniques developed in the current study to separate it from *Pipile pipile* and the use of morphology to distinguish it from *Pipile cumanensis*. Because a 100% match was not found for the UK Piping guans, a phylogenetic tree was drawn to group the species and provide further evidence for species assignment. This tree can be seen in figure 5.17 and shows the Trinidad egg sample to group with the reference *Pipile pipile* and all UK Piping guans to group with the voucher *Pipile cumanensis grayi*.

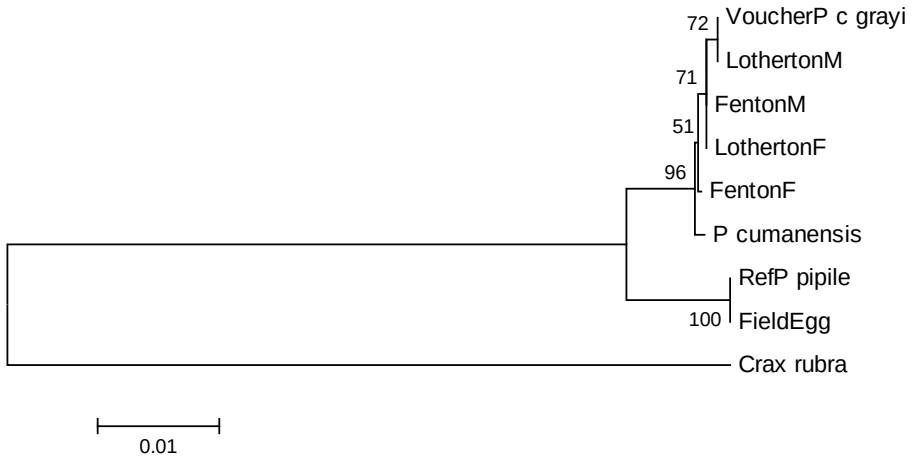


Fig 5.17: Phylogenetic tree to group birds by species drawn using 1099bp of concatenated ND2 and cyt b *sequence data. The tree was constructed with the neighbour joining method and Tamura-Nei's model of sequence evolution. Bootstrap values of an interior node test with 1000 replicates are placed on the topology.*

5.13 Conclusion

Though the species specific primers for ND2 and cyt *b* are unable to distinguish between *Pipile pipile* and *Pipile cumanensis grayi* this initial test does exclude common domestic fowl and morphologically similar species for use in field studies. Samples which produce negative results therefore do not need to undergo further processing, reducing the cost of analysis. This process is more expedient than examination by sequencing and can be performed without the need for specialised laboratory equipment.

With regard to the classification of captive birds, *Pipile pipile* can be identified or eliminated using the additional PCR-RFLP detection method. This may therefore be of use in future examinations of captive birds worldwide which are currently classed as Trinidad Piping guan. During the course of the study, additional birds have been registered on the ISIS database as Trinidad Piping guan at locations around the UK. The established methodologies could therefore examine this classification to assess the current UK captive breeding stock of the species to avoid hybridisation, especially important for stud book information should a breeding programme become established in Trinidad.

Genetic identification between *Pipile cumanensis* and *Pipile cumanensis grayi* is problematic due to the high levels of similarity between these sub-species. Further studies must be conducted to identify any possible sub-species specific SNPs and higher levels of variation within the D-loop. For classification of captive stock, morphological characteristics can be taken into account to assist species assignment. It may however be possible that the high level of genetic similarity is also seen within the nuclear genome making characteristics such as dewlap colouration susceptible to intra-species variation. Genetic identification of these sub-species is not within the scope of the current study, however methodologies have been created to confirm or refute the identity of *Pipile pipile* within field studies, investigations involving captive stock and incidences of wildlife crime in Trinidad. The methods established in the current study may be put to use in the near future as Trinidad Piping guan may still remain in the southern and central regions of the island (Naranjit, 2010b). The established

species detection tests could therefore be used to identify trace evidence from possible Trinidad Piping guan habitat. As such, the necessity of locating populations by sight, which may be problematic if a very small number of birds reside within these regions, is negated. Contact has been made with personnel working in Trinidad to highlight the possibilities of the current study for locating Trinidad Piping guan habitat without the use of distance sampling (K, Naranjit, personal communication, 4th October, 2010).

Chapter 6

6.1 General Discussion

It has been established that the current research is the first genetic study of the Trinidad Piping guan, a finding which has implications for all former genetic investigations of the species and data previously generated.

On commencement of the study, the decision to undertake non-invasive sampling posed possible problems with regard to DNA extraction but was seen as the most appropriate sample source for the species. This sampling methodology would provide viable samples, avoid disturbance to the population (Hoysak & Weatherhead, 1991; Piggott & Taylor; 2003) and utilise materials typical to that which would be obtained in future ecological or forensic studies for the purpose of species identification. The acquisition of feather samples proved the least problematic with regards to collection and export as no training or additional equipment was required. Researchers based in Trinidad are not directly connected to the University for the purpose of sample collection and therefore the study was reliant on the opportunistic collection of biological material by members of the Pawi Study Group. Although samples underwent heat treatment or ethanol washes prior to export, these conditions were essential for safe transport and did not hinder the success of DNA extraction. Feather samples are extremely resilient and are therefore unlikely to be damaged in transit, as can sometimes be the case with samples such as blood exported in glass vials. In the current study, all samples from live birds (except those received from Fenton Barns) were obtained by non-invasive feather sampling. Additionally, samples of one individual of the Trinidad Piping guan originated from egg and embryo tissues but were collected opportunistically rather than invasively. Invasive/destructive samples were only obtained when dealing with museum specimens of *Pipile cumanensis grayi*.

Feather persistence is dependant upon a number of factors (Booms et al., 2008) but this sample type is probably the most reliable source of genetic material

which is not collected immediately after deposition. The collection of tissues and faecal matter after a number of days can result in poor DNA quantities (Oehm et al., 2011) whereas feathers can be collected weeks after moulting and provide material adequate for genetic study. This is due in part to the protection provided by the keratin structure of the feather quill (Bayard de Volo et al., 2008) which is resistant enough to undergo a thorough wash-step before DNA extraction is performed. Due to extensive research into feather sampling methodologies with respect to feather type, condition and growth regions (Morin et al., 1994; Segelbacher, 2002; Rudnick et al., 2007; Bayard de Volo et al., 2008; Piggott & Taylor, 2003; Hogan et al., 2007; Taberlet & Bouvet, 1991) all samples provided viable DNA for use in the current investigation utilising the inferior umbilicus and possible adherent skin cells (Horváth et al., 2005; Bayard De Volo et al., 2008). Degradation was not seen to be a limiting factor as amplification of mitochondrial genome regions between 100 - >1000bp was observed. Amplification of ND2 (not validated for species identification) and cyt *b* (validated in 2003 by Branicki et al.) selected for further study were seen to be successful for all samples obtained. In addition, a higher level of inter species variation was observed in ND2, in agreement with Sorenson et al., (1999) aiding the separation of closely related species (Sorenson, 2003). With respect to the egg and embryo samples, although going against the original decision to undertake non-invasive feather sampling, the method by which these materials were obtained was not the result of interference by researchers. Although nest materials such as eggshell membrane can be collected as a non-invasive sample, the chance of obtaining this material in future study of the Trinidad Piping guan is limited due to the canopy behaviour of the species.

The continued gradual collection of materials in the field will assist future genetic studies and may provide opportunity for a study of the remaining population to identify species diversity. This will be especially important should a captive breeding programme ever be implemented and could also provide information regarding genotype frequencies and relatedness in the remaining population. If members of the local community in Trinidad can be encouraged to become

involved with conservation of the species as mentioned in the Pawi Species Recovery Strategy (Nelson et al., 2011) moulted feathers can be collected and passed to the Pawi Study Group for further investigation.

Study between genetic material from Trinidad Piping guan specimens obtained in the current study and those utilised in the previous phylogenetic investigation of the species (Grau et al., 2005) uncovered the apparent divergence in sequence data. On comparison with data obtained from other species in the current study it was found by SNP analysis (Ogden et al., 2009) that the previously published data did not originate from a Trinidad Piping guan but matched sequence data of Gray's Piping guan (*Pipile cumanensis grayi).* This study is not the first instance of misrepresentation in genetic studies, which has been identified in other investigations (Payne & Sorenson, 2002; Linacre & Tobe, 2011). As no verification protocols are in place for sequences deposited on GenBank, they are assumed to represent the species and therefore their subsequent use in future genetic studies can lead to possible misrepresentation. The discovery that previously generated sequence data was incorrect for the species had a number of implications. Not only were the data currently available on GenBank providing false representation of the species but being the only data attainable for Trinidad Piping guan, the current study became the first true genetic investigation of the species. As seen in the study by Grau et al., (2005), samples obtained for Trinidad Piping guan were not voucher specimens. However, the material obtained in the current study more suitably represented the species than that of the previous investigation as the captive specimen used as a reference sample had undergone sufficient studies to help verify its origin (Olsson et al., 2008). In addition, the subsequent analogous sequence data with samples obtained from free-living birds further confirmed species identification. Due to findings in the current investigation, assumptions could be made that previous phylogenetic studies may have incorrectly placed the species within the Piping guan phylogeny due to misrepresentation of the species.

Using data obtained in the current study in collaboration with sequence data available on GenBank for species of which genetic samples could not be

obtained; the phylogeny of Piping guans was re-assessed. It was found that the phylogeny created differed from that which has been previously published (Grau et al., 2005) with high bootstrap support. In addition, this was the first phylogenetic tree to be generated to include both *Pipile pipile* and *Pipile cumanensis grayi,* which had only previously been assessed in separate publications (Grau et al., 2005; Frank-Hoeflich et al., 2007). Though similar in the addition of *Pipile cumanensis grayi* to the previous *Pipile pipile* placement, the appearance of Trinidad Piping guan closer to the root of the tree was unexpected. Being an island bird endemic to land which only diverged an estimated 1500 years ago (Van den Eynden et al., 2008), it would be expected that this species would be the most recent bird to undergo speciation events due to geographical isolation. What has been shown is that the individuals studied contain a haplotype which causes a branching pattern of an earlier divergence time than would be expected. One possible explanation for this is that colonized alleles are still present within the population and speciation events may have occurred prior to separation from the mainland. Similarily, due to the recent isolation of the population it is therefore possible that the haplotype found in the Trinidad Piping guan could still be present in mainland birds. This provides opportunity for further study by investigating mitochondrial haplotypes and nuclear alleles of *Pipile pipile*, *Pipile cumanensis grayi* and *Pipile cujubi* to identify unique genetic characteristics. Due to the difficulty in obtaining samples from Trinidad, it may be more suitable to conduct large scale sampling of the common Gray's Piping guan to see if the Trinidad haplotype is expressed, rather than looking for a single haplotype in a limited sample size. Diversity was not examined in the current study due to the availability of samples and timescale of the investigation. Because the nuclear genome of the Trinidad Piping guan has not undergone previous analysis, microsatellites have not been established for the species. Markers therefore need to be designed for the species or cross-species microsatellites require identification through experimentation.

Due to the replacement of *Pipile pipile* by *Pipile cumanensis grayi* in the phylogeny constructed by Grau et al., (2005), the previously published

divergence time calculated for *Pipile pipile* in (0.4 - 1.6MYA) which was inconsistent with the age of Trinidad could therefore represent the divergence time of *Pipile cumanensis grayi.* Further study is required to calculate the divergence time of *Pipile pipile* and examine the rate of substitutions within the control region (Pereira et al., 2004). In addition, this study supports the merge of *Pipile* into *Aburria*, being the earlier synonym, in concurrence with Delacour & Amadon, (1973), Pereira, (2000), Grau et al., (2005) and Frank-Hoeflich et al., (2007). However, it is unlikely that this merge will be accepted until research is conducted which utilises voucher specimens or highly credited reference samples and the resultant phylogeny provides a high level of bootstrap support. One of the main issues which resulted in the previous rejection of this merge by the South American Classification Committee in 2007 (Remsen et al.) was the lack of the voucher specimens. Genetic research is often heavily reliant on reference samples but there was some element of support for this rejection in the discovery that one species included in the proposal to merge these two synonyms was misrepresented. Most importantly, the material representing the species *Aburria aburri* must have a high level of confidence with respect to species identification and should originate from a voucher specimen if possible (Leeton et al., 1993; Bates et al., 2004). As well as misrepresenting the species in the phylogeny, incorrect classification on GenBank results in false species identification when utilising search databases such as BLAST (Altschul et al., 1997). This database is perhaps the most frequently used method of providing genetic identification in research of an unknown sample and therefore should a true representative of *Pipile pipile* be examined using BLAST, species identification would not have been possible. On publication of data obtained in the current study, sequence information will be deposited in GenBank for use in species identification and future genetic research.

To enable rapid species detection, methods were created using species specific primers and PCR-RFLP to identify samples originating from Trinidad Piping guan before confirmation with nucleotide sequencing to assist the validity of results (Wen et al., 2010). Because of the limited genetic variation between

Piping guans, the species specific primers provide a method of eliminating species outside of the Genus to exclude morphologically similar species such as the black vulture, with PCR-RFLP then providing species detection. By using multiple enzymes this helps alleviate the possibility of mutation occurring in the recognition sites and providing a false negative. It is unlikely that mutation would occur at all three sites and therefore any positive result should undergo further investigation. The method of PCR-RFLP within wildlife forensics is being replaced by more advanced technologies but the cost of performing these tests is far greater than RFLP analysis. Conservation studies looking at species identification are well suited to this method as it can be performed within most standard laboratories and the results easy to interpret. When studying a species of which little is known regarding genetic diversity it is also beneficial to establish a method which can allow for some level of mutation whilst still providing interpretable results. This methodology has been seen in previous publications of closely related species such as tigers in which species primers can define tigers from other cat species (Wan & Fang, 2003) but would not allow sub-species identification (Kitpipit, Linacre & Tobe, 2009). When the level of sensitivity in species specific primers is insufficient due to minimal genetic variation, RFLPs can enable the separation of closely related species based on single point mutations (Ferreira, Martins, Ditchfield & Morgante, 2005).

These relatively cheap methods for standard laboratories can be conducted in order to minimise costs of an investigation and avoid the study and sequencing of non-target samples. Detection methods such as this have implications for forensic investigations in the identification of wildmeats currently served at BBQs in Trinidad and trace samples such as blood found on the property of persons suspected of illegal hunting. The most prevalent threat to the species besides the possibility of pathogenic disease spread by domestic fowl is hunting. Trinidad is not an island which is reliant on bushmeats as an important source of protein, but hunting of the Trinidad Piping guan is widespread and birds are taken opportunistically as a rare meat whilst hunting other animals. Previously, hunters had to be caught in possession of a bird or full carcass to prove breach

of the hunting ban. However, if the methods established in the current study were implemented, species identification would be possible by trace evidence or to identify meats of unknown origin. Although penalties can be light and the funds available to conduct these kinds of genetic investigation for wildlife crime may be limited, it may be possible that development of these techniques alone would provide a deterrent and help to alleviate the continued pressure of hunting. As these techniques can be put into force by any standard laboratory, samples can be examined for the Trinidadian police force, maintaining continuity of evidence should any investigation ever reach court (Cooper et al., 2009). If sample collection continues in Trinidad, these methods can also be used to identify feathers which originated from the Trinidad Piping guan before further genetic research is conducted. Those which do not originate from the species can be discarded and those providing positive results can then receive an identification number and information about the sample (such as location) can be entered into a database. By creating a database of all known Trinidad Piping guan samples, this will assist future researchers and move towards a central repository of information. This issue was discussed in the Pawi Species Recovery Strategy (Nelson et al., 2011) to improve networking and co-ordination of scientific research.

By recording the collection sites of feathers it would therefore be possible to investigate habitat range of the species. Though it is thought that the Trinidad Piping guan is restricted to habitat in the Northern Ranges of the island, a study of the central and Southern regions has not been conducted for over a decade (Hayes et al., 2009). Identifying remaining populations by sight is problematic as the terrain of possible habitat makes it extremely difficult to locate and identify the elusive species. This was demonstrated in the previous assessment of population size abandoned after the pilot study of known habitat regions resulted in no individuals being identified (Keane et al., 2005). If however, possible trace evidence such as feathers could be obtained opportunistically then populations of the species could be genetically identified without direct observation of the birds. As flight of the species is limited there is also no risk of identifying a

feather which was dropped in flight in a region not populated by the species. This would allow researchers to provide protection for habitat and further understand the distribution of the species on the island without having to observe and possibly reveal the specific location of the birds. Genetic detection methods have multiple applications in the Pawi Species Recovery Strategy (Nelson et al., 2011) with regard to spatial monitoring, identifying ranges of habitats and the species tolerance to habitat change by locating birds in areas outside of the Northern range of Trinidad. The collection of moulted feathers for genetic study also enables the involvement of local communities. This not only provides an avenue for the locals to get involved with conservation of the islands only endemic bird but also increases the task force of persons collecting trace evidence for analysis.

According to the ISIS database, the Trinidad Piping guan is held in captivity within bird parks around the world. However, on further investigation of this claim it was found that a related breeding population in the UK of birds held at Fenton Barns in East Lothian, Cotswold Wildlife Park and Gardens in Oxfordshire and Lotherton Bird Garden in Leeds were misclassified. After investigation using methods devised in the current study, it was found that these birds grouped with Gray's Piping guan (*Pipile cumanensis grayi)* as seen in the previous misclassification of published data. The origin of the misidentified *Pipile pipile* samples from Grau et al., (2005) were taken from a bird held at a private aviary in Mexico. Classification of this bird was most likely based on morphological examination, the same method used to identify birds at the UK parks which were investigated. This demonstrates the ease in which the Trinidad Piping guan can be misidentified due to morphological similarities. Since this research was conducted, the ISIS database has been updated and two further sites in the UK now claim to house Trinidad Piping guan, these are Blackpool Zoo and the Harewood Bird Garden in Leeds. It is recommended that classification of these birds and others registered worldwide on ISIS are verified using the genetic methodologies and data established in the current study.

The present study has demonstrated the beneficial applications of remotely collected non-invasive samples. This sampling methodology is therefore acceptable for use in the study of the species with regards to issues such as minimising disruption to the population, collection and export of materials and the quality of genetic material which can be obtained. The current research has highlighted the previous misrepresentation of the species and provided methodologies and genetic data for the purpose of species identification. In addition, the data acquired has allowed the re-assessment of the current phylogenetic placement of the Trinidad Piping guan, altering the species evolutionary history.

It is hoped that the techniques devised in this study, as well as the culmination of information regarding the species, will be of use to persons such as the Pawi Study Group, Trini Eco-Warriors as well as future researchers in studying and protecting the remaining population of the Trinidad Piping guan.

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Appendices

I - VII

Appendices I

Sequence data obtained in the current study from the reference Trinidad Piping guan (*Pipile pipile*)

ND2

TCCATGCAAAGTTAATTACAACCTTAAGCCTCTTTGCAGGAACAACCATTGTAATCTCAAGC
AATCACTGAATGGCAGCCTGGACAGGCCTAGAAATTAACACCCTAGCCATCATCCCCCTCA
TTGCCAAATCCCATCACCCGCGAGCAATCGAGGCGACAATCAAATACTTCTTAACACAATCC
ACTGCATCAGCCTTAATCCTTTTCTCAAGCATAATCAATGCCTGACTCACTGGCCAATGAGA
CATCACCCAGCTAAACCACCCCCTCCCGTGCCTTATACTAACAACAGCAATCGCAATCAAA
CTTGGCCTAGCCCCATTCCACTTTTGATTTCCAGAAGTACTCCAAGGATCGCACCTAACCAC
TGCCCTATTACTCTCAACACTAATAAAATTCCCCCCACTCACACTCCTTCTAGTAACGTCAAA
ATCACTAAACCCTATCCTGCTTATTACCCTAGCTATCACCTCAATAGCACTGGGAGGCTGAA
TAGGCCTCAATCAAACACAAACACGAAAAATTCTAGCCTTCTCATCCATTTCCCACTTAGGA
TGAATAACCCTAGTCACCATCTATAGCCCCAAACTCAGCCTACTAACCTTCCTCCTCTATAC
AGTAATAACAACAACCGTATTCCTCTCACTAAACATAATCAAAGTCCTAAAACTATCAACTAT
GCTAATCTCATGAACAAAAACTCCCACCCTAAATGCAGCCCTAATACTCACCTTACTTTCTCT
AGCAGGCCTTCCCCCATTAACTGGCTTCATACCCAAGTGATTAATCATCCAAGAACTCACCA
AACAAGAAATAACCCCTGCCGCTACAATCGCCGCCATGCTATCACTCCTGGGCCTATTCTTT
TACCTTCGCCTCGCATACCACTCAACAATCACTCTCCCACCTAACTCCTCCAATCACATAAA
AACATGGTATCTCCGTAAAACACCAAACACCTTCACCGCCATCCTGACTTCACTATCAATCT
CCCTCCTGCCCCTCTCCCCTATAATTCTCACCATGGTC

1031bp

WANCY cluster (tRNA Trp - tRNA Ala - tRNA Asn - tRNA Cys - tRNA Tyr)

TAGAAACTTAGGATAACCCAAACCCTCCCAAACCGGAGGCCTTCAAAGCCTCAAATAAGAG
TTAAACCCTCTTAGTTTCTGCAACACTAAGGCCAACAGGATATTAACCTGTATCTCCTGAAT
GCAAATCAAATGCTTTAATTAAGCTAAAGCCTTACCTAGGCAGATGGGTCTTGATCCCATGT
AACTCTAGTTAACAGCTAAATGCCTTACCTCCTGGCTCCTGCCTACAAAGACCCTGGCACA
CCTTAATGTACATCATTGAGCTTGCAACTCAATATGAATTTCACCACAGGGCCGATAAGAAG
AGGAATTGAACCTCTGTAAAAAGGACTACAGCCTAACGCTTACGCCACTCAGCCATCTTAC
CT

371bp

COI

GTGACCTTCATCAATCGATGATTATTCTCAACTAACCATAAAGACATCGGCACCCTCTACTT
AATTTTTGGTACATGAGCAGGCATAGTAGGCACCGCACTAAGCCTGCTAATTCGCGCAGAG
CTTGGCCAACCAGGAACCCTACTAGGAGACGATCAAATCTACAACGTAATCGTCACAGCCC
ATGCCTTCGTCATAATCTTCTTCATAGTCATACCAATCATAATTGGCGGCTTTGGAAACTGA
CTAGTCCCCCTCATAATCGGCGCGCCCGACATAGCATTCCCACGAATAAACAACATAAGCT
TCTGACTCCTTCCCCCATCCTTTCTTCTTTTACTAGCCTCATCCACCGTAGAAGCTGGAGCC
GGAACAGGATGAACTGTCTACCCCCCCTTAGCAGGAAACCTAGCCCACGCTGGCGCTTCA
GTAGACCTGGCCATCTTCTCCCTTCACCTAGCAGGTGTCTCCTCTATCCTAGGGGCAATCA
ACTTTATTACTACCGCCATCAACATAAAACCCCCAGCCCTCTCACAATACCAAACCCCTCTA
TTCGTATGATCAGTCCTTATCACTGCTATCCTACTTTTATTATCCCTCCCCGTCCTAGCAGCT
GGCATCACCATACTCCTTACTGACCGTAATCTTAATACCACATTCTTCGACCCAGCCGGGG
GCGGAGACCCAGTTCTATATCAACACCTCTTCTGATTCTTTGGTCACCCAGAAGTTTACATC

CTCATTCTCCCTGGCTTTGGGATCATTTCCCACGTAGTAGCATACTACGCTGGCAAAAAAGA
ACCATTCGGCTACATAGGAATAGTATGAGCAATACTATCAATCGGATTTCTTGGCTTCATCG
TATGAGCCCACCATATATTCACAGTAGGTATAGACGTAGACACTCGAGCTTACTTCACATCT
GCCACTATAATCATTGCCATCCCAACTGGCATTAAAGTCTTCAGCTGACTTGCCACCCTGCA
TGGAGGTACAATTAAATGAGACCCTCCTATACTATGAGCCCTAGGATTTATCTTCCTATTCA
CCATTGGCGGCCTAACAGGAATTGTCCTCGCAAACTCTTCATTAGACATCGCCTTGCACGA
CACATACTATGTAGTAGCCCATTTCCACTACGTTCTCTCTATGGGGGCAGTCTTTGCCATTC
TAGCTGGGTTTACCCACTGATTCCCCCTATTCACAGGCTTTACCCTCCACCCCACATGAACC
AAGACCCACTTTGGAGTCATATTTGCGGGAGTAAACCTAACCTTCTTCCCACAGCACTTCCT
AGGCCTAGCGGGCATACCCCGCCGATACTCTGACTACCCAGACGCCTACGCACTATGAAA
CACCCTATCCTCCATCGGCTCCCTAATCTCAATAACAGCCGTAATTATACTAATATTTATCGT
CTGAGAGGCTTTCTCCTCAAAACGTAAAGTTCTACAGCCCGAATTAACCACTACCAACATTG
AATGAATTCATGGTTGCCCACCCCCATACCACACCTTCGAAGAACCAGCCTTCGTCCAAGT
CCAAGAA

1548bp

Cyt *b*

ATGGCCCCCAACATCCGAAAATCTCACCCTCTGCTAAAAATAATCAACAACTCTCTAATCGA
CCTCCCCGCCCCATCAAACATCTCCGCCTGATGAAACTTTGGATCCTTACTGGCAATCTGC
CTTACAACCCAAATCCTCACCGGCCTCCTATTAGCCATACACTACACCGCAGATACCACTCT
CGCCTTCTCCTCCGTAGCCCACACATGCCGAAACGTCCAATACGGCTGGCTAATCCGCAAC
CTACATGCAAACGGCGCCTCATTCTTCTTTATCTGCATCTACCTCCATATTGGTCGCGGCTT
CTACTACGGCTCATACCTCTACAAAGAAACTTGAAACACAGGAGTAATCCTCCTACTAGTAC
TTATAGCAACTGCTTTCGTAGGATATGTTCTCCCATGAGGACAAATATCATTCTGAGGAGCC
ACCGTTATCACCAACCTATTCTCAGCCATCCCCTATATCGGACAAACCCTAGTAGAATGAGC
TTGAGGGGGATTTTCAGTTGACAACCCAACCCTAACACGATTCTTCGCCCTACACTTTTTAC
TTCCATTTGCAATCGCGGGCATTACCGTAATTCACCTCACCTTCCTCCACGAGTCCGGCTC
AAACAACCCCCTAGGACTCACATCCGATTGCGACAAAATCCCCTTCCACCCCTACTTCTCC
CTAAAAGATATTCTAGGCTTCTCACTCATATTCATCCCCCTCCTAACACTTGCCTTCTTCAAC
CCAAATCTTCTAGGGGACCCAGAAAACTTCACACCAGCAAATCCCCTAGTCACCCCTCCAC
ATATTAAGCCTGAATGATACTTCTTATTTGCATACGCCATTCTACGCTCAATCCCCAACAAGC
TTGGAGGGGTCCTCGCACTAGCAGCCTCAGTACTAATCCTCTTCCTCATCCCCCTTCTACA
CAAATCCAAACAACGAGCAATGACATTCCGCCCCTTCTCTCAACTCCTATTCTGACTTCTAA
CTGCCAACCTCCTAATCCTAACATGAGTGGGCAGCCAACCAGTAGAACACCCATTCATCAT
CATCGGACAACTCGCTTCCCTCACCTATTTCACCATCTTACTTCTCCTCTTCCCAATCACCG
GAGCCCTAGAAAACAAAATACTTCGCCAC

1140bp

tRNA Thr & tRNA Pro

TAAAATACTCTAATAGTTTACAACAAAACATTGGTCTTGTAAACCAAAGATCGAAGACTACAA
CCTTCTTAGAGT **75bp**
GACTCAGAAAAAAAGGACTCAAACCTCTTTCTCCGGCTCCCAAAGCCGGTATTTTCCAATAA
ACTATTTTCTGACCCCCCTAAC **84bp**

ND6

CGCCCGAATTGCCCCCCGAGATAACCCCCGCACAACCTCTAACACGACAAACAACGTTAAC
AACAACCCCCACCCAGCCACCAAAAACAACCCTACACCACAAGAATAAAACACAGCCACAC
CACTAAAATCCAACCGTGTAAAAAACACCCCTCCACCATCAACAGTACTTACCGCAACTTTC
CAAAACTCAACAAACCCCCCAAAAACAACCACCACAAGCACTAACAGAACAAAACTCAACC
CATACCCTACCACCCGCCAATCCCCCCAAGCCCCAGGAAACGGGTCCGCCGCCAGCGACA
CTGAATAGACAAAAACCACCAACATCCCACCTAAATACACCATAAACAACACCAGAGACACA
AAAGAGACCCCCAAACTCAACAACCACCCACACCCCGCCACAGACCCCAACACCAACCCC

ACTACCCCATAATACGGAGAGGGGTTAGATGCCACAGCTAAAGCCCCTAACACAAAACTAA
ACCCAAGAAAAACTACAAAATAAGTCATATA **519bp**

tRNA Glu

CTCCCACCTGGATTTTACCCAAGACCTACGGCCTGAAAAGCCATCGTTGTTCTCAACTATG
GGAGCAACT **70bp**

D-loop

AATTTTTAACCTAACCCTCCTACTAAGCACATCTCTCTCTAGCAAGGGCCCCCCCCCCTACC
C **Missing section of D-loop - 404bp**
CTACGTGACTAGCTTCAGGCCCATTCGTTCCCCCTAAACCCTCGCCCCTCTTGCTCTTTTGC
GCCTCTGGTTCCTATATCAGGGCCATCCTTCTCCTACTCCCTATCACTATGCTTTTCACGAG
GCATCTGGTCGGAGAATACCCACCATTTTAGTCCGTGATCGCGGCATCTTATCTCTTCTCTG
CTATTGGTTCCCTTTTTTTTCTGGGGCTTCTTCACAGCTGGCATTTCCAGTGCCGTTGCGGA
GTGCACACAATCTAGGTCTGGACATCTCCTGGTTTGCGTCCTATTCTAGACCTCAGGCGTC
CCTCAATGAGACGGTTTGCGTGTATGGGGAATCATTTTGACACTGATGCACTTTGGATCGC
ATTTGGTAATGGCTCTTCCACCCCGCCCAAACATGGTGCTATTTAGTGAATGCTTGACGGG
CATATTTTTGCAAATTTTCACTTCCTCTATTTTTCTAACAAAACTAGGAAGTTTTCCTAACTTT
TTTTTCGATCATCAACAAACGTTTTACAAAAAAATTTATTTGCATTTAAAAACAGCGCTAAGAT
TCCCTTAACACCATCCAAAAACGTTTGCATTATATACATACATTTGTTACACACCAAATTTATT
AGAGAAACTCCACTACCAAAAACAACCACCACAAACATACATTTTCTTTCTTTTCCAATCCTG
GACAAACCGACCAAAAACAAACTATCACCCAGCTCCCATAGCTTAGCTCAAAGCACGGCAC
TG **812bp**

Appendices II

All primers used throughout the investigation for the purpose of universal amplification and the generation of designed primers showing main author and year of original publication, separation does not show reading frame.

CODE	JOURNAL	Region	SUFFIX	L STRAND	SUFFIX	H STRAND
A	Sorenson (1999)	D-Loop	**L436**	TCT CAC GAG AAC CGA GCT AC	**H774**	CCA TAT ACG CCA ACC GTC TC
B	Sorenson (1999)	D-Loop	**L436 REVI**	CCT CAC GAG AAA YCA GCA AC	**H774**	CCA TAT ACG CCA ACC GTC TC
C	Sorenson (1999)	D-Loop	**774L REV**	GAG ACG GTT GGC GTA TAT GG	**H1251**	TGG CAG CTT CAG TGC CAT GC
D	Sorenson (1999)	D-Loop	**774L REV**	GAG ACG GTT GGC GTA TAT GG	**H1251 REVI**	TCT TGG CAT CTT CAG TGC CRT GC
E	Grau (2003)	D-Loop	**DloopL**	TTG TTC TCA ACT ACG GGA AC	**DloopH**	GTG AGG TGG ACG ATC AAT AAA T
F	Sorenson (1999)	ND2	**L5143**	GAA CCT ACA CAR AAG RGA TCA AAA C	**H5766**	GGA TGA GAA GGC TAG GAT TTT KCG
G	Sorenson (1999)	ND2	**ND2 5766L**	CGW AAA ATC CTA GCC TTC TCA TCC	**H6313**	CTC TTA TTT AAG GCT TTG AAG GC
W	Sorenson (2003)	ND2	**L5216**	GGC CCA TAC CCC GRA AAT G	**H6313(ii)**	ACT CTT RTT TAA GGC TTT GAA GGC
1	Sorenson (2003)	ND2	**L5216**	GGC CCA TAC CCC GRA AAT G	**H5766(ii)**	RGA KGA GAA RGC YAG GAT YTT KCG
2	Sorenson (2003)	ND2	**L5758**	GGN GGN TGA ATR GGN YTN AAY CAR AC	**H6313(ii)**	ACT CTT RTT TAA GGC TTT GAA GGC
MND2	Designed	ND2	**Lc5568**	AGG ATC GCA CCT AAC CAC TGC C	**Hc5608**	GTA GGC TGA GTT TGG GGC TA
H	Kocher (1989)	cyt *b*	**L14841**	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	**H15149**	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A
R1	Hebert (2004)	COI	**BirdF1**	TTC TCC AAC CAC AAA GAC ATT GGC AC	**BirdR1**	ACG TGG GAG ATA ATT CCA AAT CCT GG
R2	Hebert (2004)	COI	**BirdF1**	TTC TCC AAC CAC AAA GAC	**BirdR2**	ACT ACA TGT GAG ATG ATT

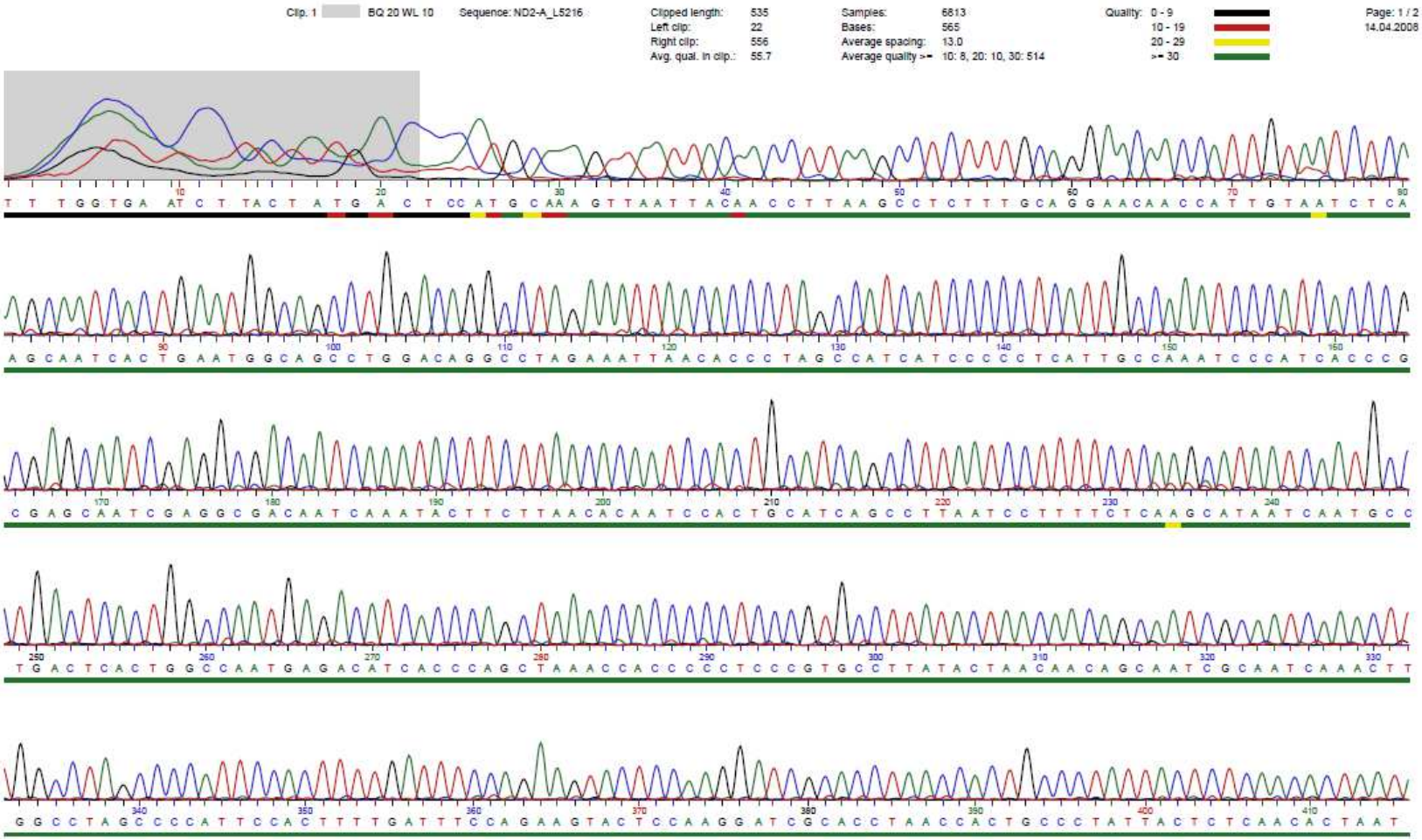
				ATT GGC AC		CCG AAT CCA G
R3	Hebert (2004)	COI	**BirdF1**	TTC TCC AAC CAC AAA GAC ATT GGC AC	**BirdR3**	AGG AGT TTG CTA GTA CGA TGC C
SS1	Designed	COI	**L0014**	CCC ATG CCT TCG TCA TAA TC	**H0472**	GGT CAG TAA GGA GCA TGG TGA
SS2	Designed	COI	**L0334**	ACC GCC ATC AAC ATA AAA CC	**H1080**	TCC AAA GTG GGT CTT GGT TC
C1	Pereira (2004)	COI	**LTyr**	TGT AAA AAG GWC TAC AGC CTA ACG C	**H8205**	GGT TCG ATT CCT TCC TTT CTT G
C2	Tavares (2008)	COI	**LTyr**	TGT AAA AAG GWC TAC AGC CTA ACG C	**COI907aH2**	GTR GCN GAY GTR AAR TAT GCT CG
I1	Tavares (2008)	COI	**COIaRt**	AAC AAA CCA CAA AGA TAT CGG	**COI748Ht**	TGG GAR ATA ATT CCR AAG CCT GG
I2	Tavares (2008)	COI	**LTyr**	TGT AAA AAG GWC TAC AGC CTA ACG C	**COI745h2**	ACR TGN GAG ATR ATT CCR AAN CCN G
Cb	Gupta (2005)	cyt *b*	**mcb398**	TAC CAT GAG GAC AAA TAT CAT TCT G	**mcb869**	CCT CCT AGT TTG TTA GGG ATT GAT CG
	Designed - NOT used together	COI	**F794 C4**	GAA CCA TTC GGC TAC ATA GG	**R859 C3**	ATG AGC CCA CCA TAT ATT CAC A
b1	Kimball (1999)	cyt *b*	**L14851**	TAC CTG GGT TCC TTC GCC CT	**H15670**	GGG TTA CTA GTG GGT TTG C
b2	Kimball (1999)	cyt *b*	**L15311**	CTC CCA TGA GGC CAA ATA TC	**H16065**	TTC AGT TTT TGG TTT ACA AGA C
	Designed - NOT used together	COI	**L674 C6**	GGA GAC CCA GTT CTA TAT C	**H763 C5**	GCG TAG TAT GCT ACT ACG TGG
d1	Xiao (2006)	D-loop	**ctb13354F**	GCT GAC TAC TCC GCA ACC TAC ACG CAA ATG	**12S256R**	GAA TGT AGC CCA TCT CTT CCA CCT CAT AGG
d2	Xiao (2006)	D-loop	**ND6527F**	AAG CCG CCG TTA ACT CAC CC	**ctb221R**	GTG GTG GGA GCA TTA GGA TT
d3	Xiao (2006)	D-loop	**ctb1592F**	GGT GTA GGA GGG GGG AAA	**12S211R**	CCT TGG GTG TTC TGT GGT GA
b3a	Kocher (1989) and Kimball (1999)	cyt *b*	**L14841**	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	**H15670**	GGG TTA CTA GTG GGT TTG C
b3b	Kimball (1999)	cyt *b*	**L14731**	ATC GCC TCC CAC CTR ATS GA	**H15298**	CCT CAG AAT GAT ATT TGT CCT CA
b4	Kimball (1999)	cyt *b*	**L14731**	ATC GCC TCC CAC CTR ATS	**H15670**	GGG TTA CTA GTG GGT TTG

				GA		C
b5	Kocher (1989)	cyt *b*	**L14724**	CGA AGC TTG ATA TGA AAA ACC ATC GTT G	**H15149**	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC
b6	Thomassen (1989) and Designed	cyt *b*	**ND5**	TAG CTA GGA TCT TTC GCC CT	**cytbDesi**	TGT AGG TTG CGG ATT AGC CAG C
b7	Sorenson (1999) and Designed	cyt *b*	**L14080**	TCA ACN CAY GCM TTC TTY AAR GC	**cytbDesi**	TGT AGG TTG CGG ATT AGC CAG C
W1	Designed	WANCY	**L6182**	TGG TAT CTC CGT AAA ACA CC	**H6735**	GCC TAC TAT GCC TGC TCA TGT A
IQM1	Sorenson (2003)	IQM	**L4500**	GTN GCM CAA ACN ATY TCH TAY GAA G	**H5201**	CCA TCA TTT TCG GGG TAT GG
IQM2	Sorenson (2003)	IQM	**L5143**	GAA CCT ACA CAR AAG RGA TCA AAA C	**H5766**	RGA KGA GAA RGC YAG GAT YTT KCG
12a	Kocher (1989)	12S	**L1091**	AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT	**H1478**	TGA CTG CAG AGG GTG ACG GGC GGT GTG T
BB	Designed	D-loop	**L465**	GCG CCT CTG GTT CCT ATA TCA	**H839**	AAG CAT TCA CTA AAT AGC ACC A
ND6i	Designed	ND6	**Lcytb**	CAT GAG TGG GCA GCC AAC CAG T	**Hdloop**	GCT AGA AAG ATG CAT GTC CGT C
12b	Pereira (2000a)	12S	**L1537**	AAT CTT GTG CCA GCC ACC GCG G	**12SEND**	GTG CAC CTT CCG GTA CAC TTA CC
16a	Pereira (2000a)	16S	**16SAR**	AAG CCW ANC GAG CYG GGT GAT AGC TGG	**16SBR**	CAT AGA TAG AAA CCG ACC TGG
d4	Designed	D-loop	**L_ND6**	ACC AGA GAC ACA AAA GAG ACC C	**H_CR**	GTG GGT ATT CTC CGA CCA GAT G
C7	Hebert (2003)	COI	**LCO1490**	GGT CAA CAA ATC ATA AAG ATA TTG G	**HCO2198**	TAA ACT TCA GGG TGA CCA AAA AAT CA
UGb	Robinson (2006)	cyt *b*	**L15714**	TTT CTA TTC GCC TAT GCC AT	**H15923**	ACT GGT TGG CTT CCG ATT CA
F1	Sorenson (2003)	ND2	**L5216**	GGC CCA TAC CCC GRA AAT G	**H5766**	GGA TGA GAA GGC TAG GAT TTT KCG
3	Designed & Sorenson	ND2	**L508**	CGA RAR ATY CTA GCC TTC TCR TCY A	**H6313(ii)**	ACT CTT RTT TAA GGC TTT GAA GGC
4	Designed & Sorenson	ND2	**L503**	ACV CGA RAR ATY CTA GCC TTC TC	**H6313(ii)**	ACT CTT RTT TAA GGC TTT GAA GGC
5	Designed & Sorenson	ND2	**L5758**	GGN GGN TGA ATR GGN YTN	**H6681**	GGT ATA GGG TGC CRA TRT

				AAY CAR AC		CTT TGT G
6	Designed & Sorenson	ND2	**L503**	ACV CGA RAR ATY CTA GCC TTC TC	**H6681**	GGT ATA GGG TGC CRA TRT CTT TGT G
7	Designed & Sorenson	ND2	**L508**	CGA RAR ATY CTA GCC TTC TCR TCY A	**H6681**	GGT ATA GGG TGC CRA TRT CTT TGT G
d5	Designed	D-loop	**L2ND6**	TAC CCC ATA ATA CGG AGA GG	**H2CR**	GAG ATG TCC AGA CCT AGA TT
PS	Designed	ND2	**Lpipile**	GCA GCC CTA ATA CTC ACC TTA CTT	**Hpipile**	GTA TGC GAG GCG AAG GTA AA
PSii	Designed	ND2	**Lpipile**	GCA GCC CTA ATA CTC ACC TTA CTT	**H2Pipile**	AAG AAT AGG CCC AGG AGT GAT AGC
CS	Designed	ND2	**LCPipile**	GCA GCC CTA ATA CTC ACC CTA CTC	**Hpipile**	GTA TGC GAG GCG AAG GTA AA
b8	Kornegay (1993)	cyt *b*	**L14990**	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	**H15696**	AAT AGG AAG TAT CAT TCG GGT TTG ATG
b9	b3 and b8 mix	cyt *b*	**L14990**	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	**H15670**	GGG TTA CTA GTG GGT TTG C
b10	b3 and b8 mix	cyt *b*	**L14841**	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	**H15696**	AAT AGG AAG TAT CAT TCG GGT TTG ATG
PS3	Designed	cyt *b*	**Lpawi**	CGG ACA AAC CCT AGT AGA ATG AGC TTG AGG G	**Hpawi**	C ATT GCT CGT TGT TTG GAT TTG TGT AGA
PS4	Designed	cyt *b*	**LPawi2**	CCT AGT AGA ATG AGC TTG AGG G	**HPawi2**	GCT CGT TGT TTG GAT TTG TGT AGA
PS5	Designed	cyt *b*	**LPawi3**	AAA CGT CCA ATA CGG CTG G	**HPawi3**	CTA CTA GGG TTT GTC CG

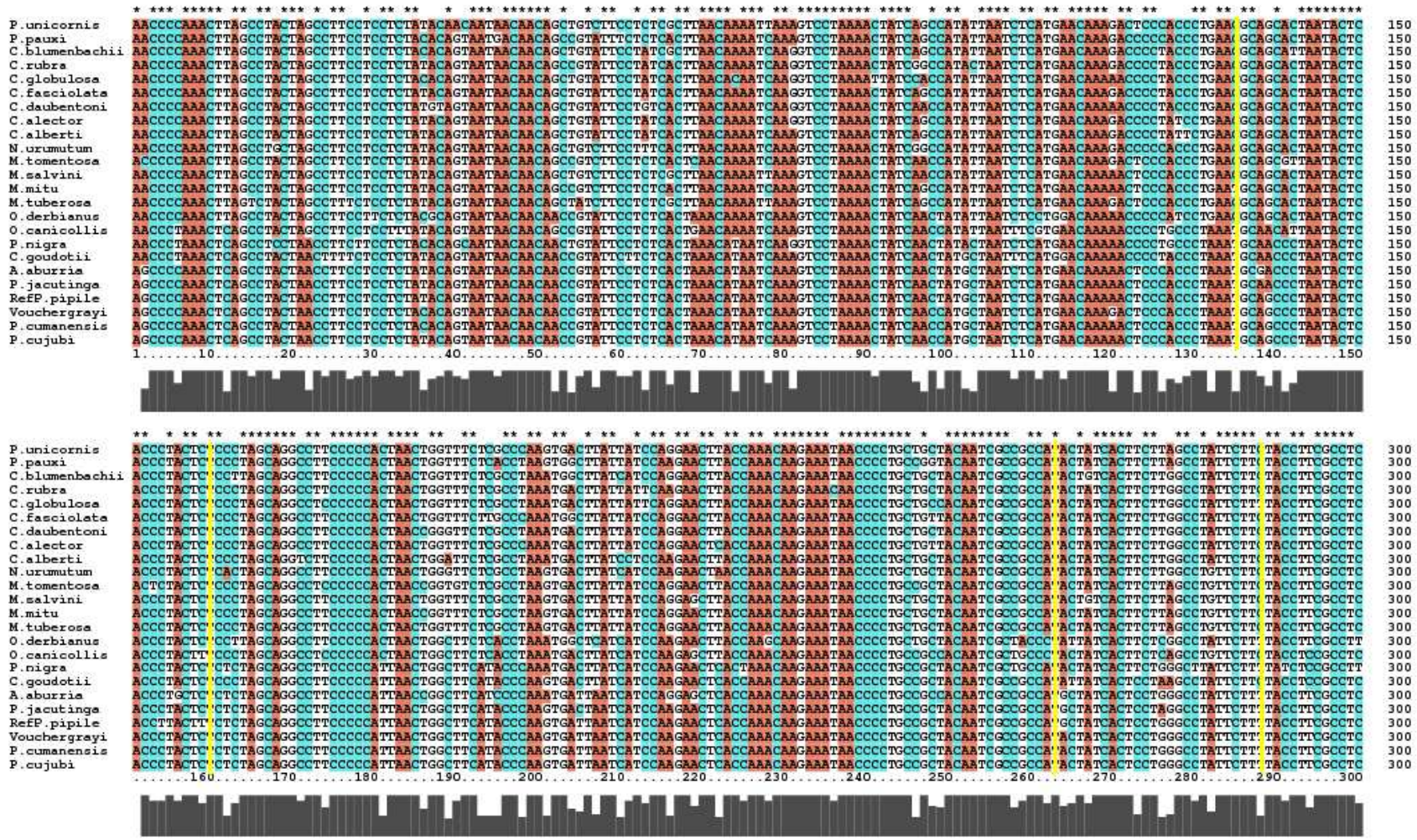
Appendices III

Example of electropherogram obtained from MWG Eurofins



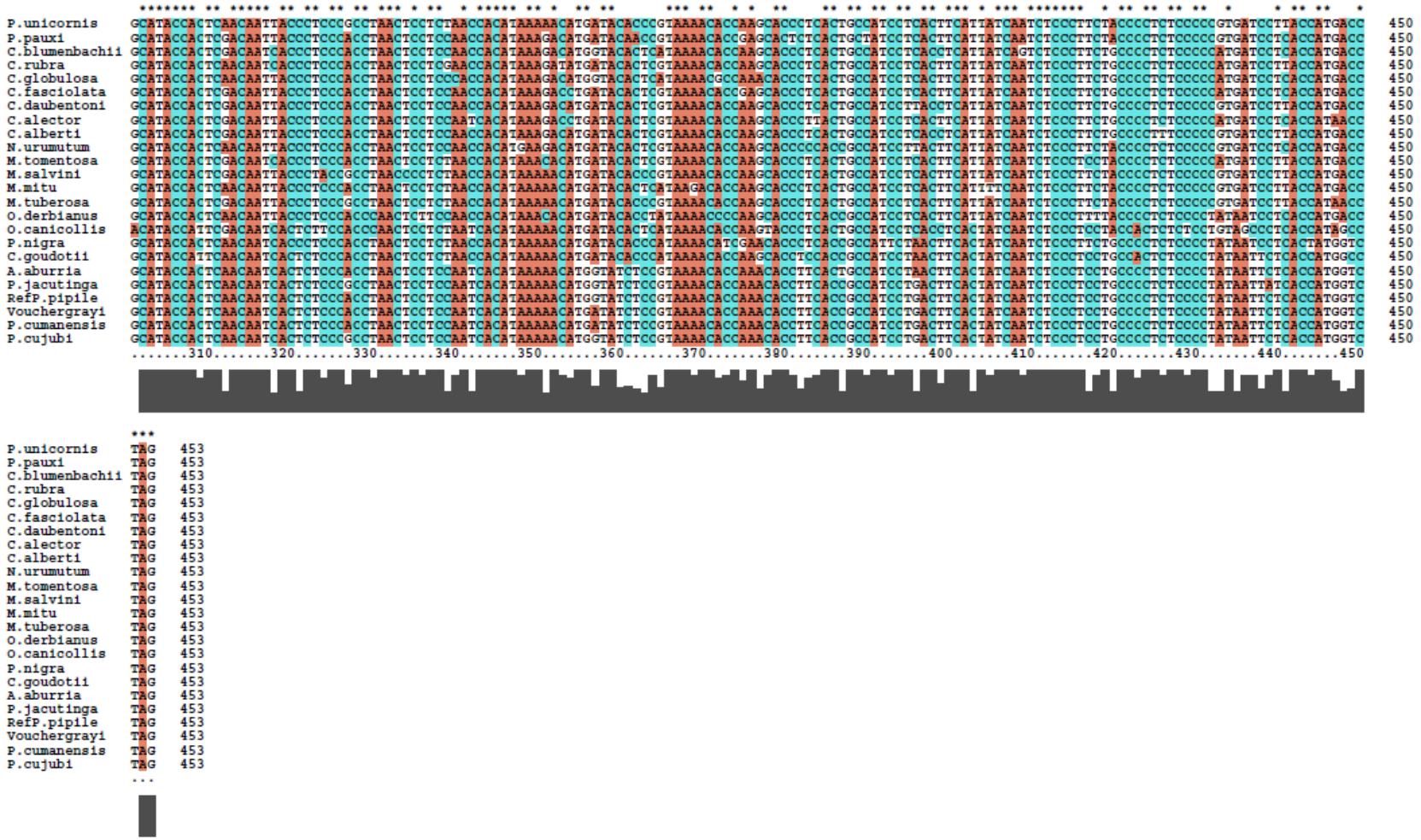
Appendices IV

ND2 alignment: Page 1 of 2



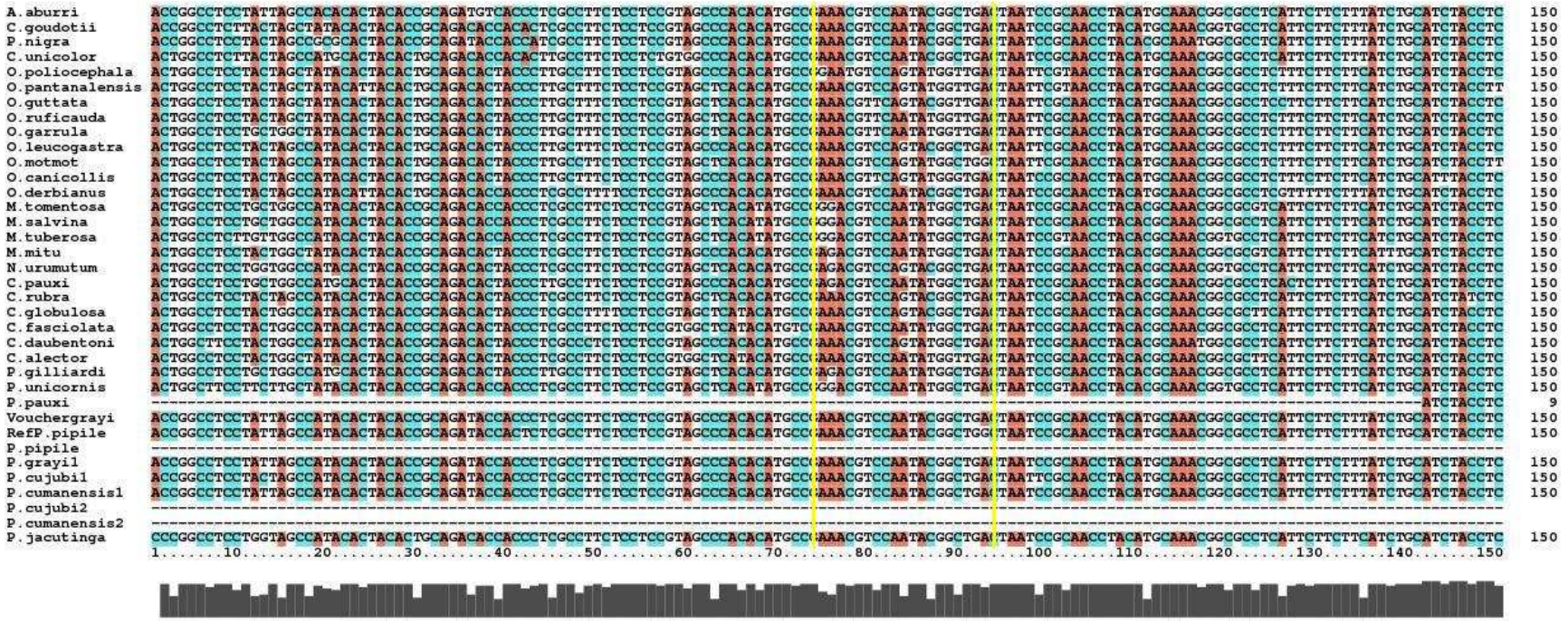
Appendices IV

ND2 alignment: Page 2 of 2

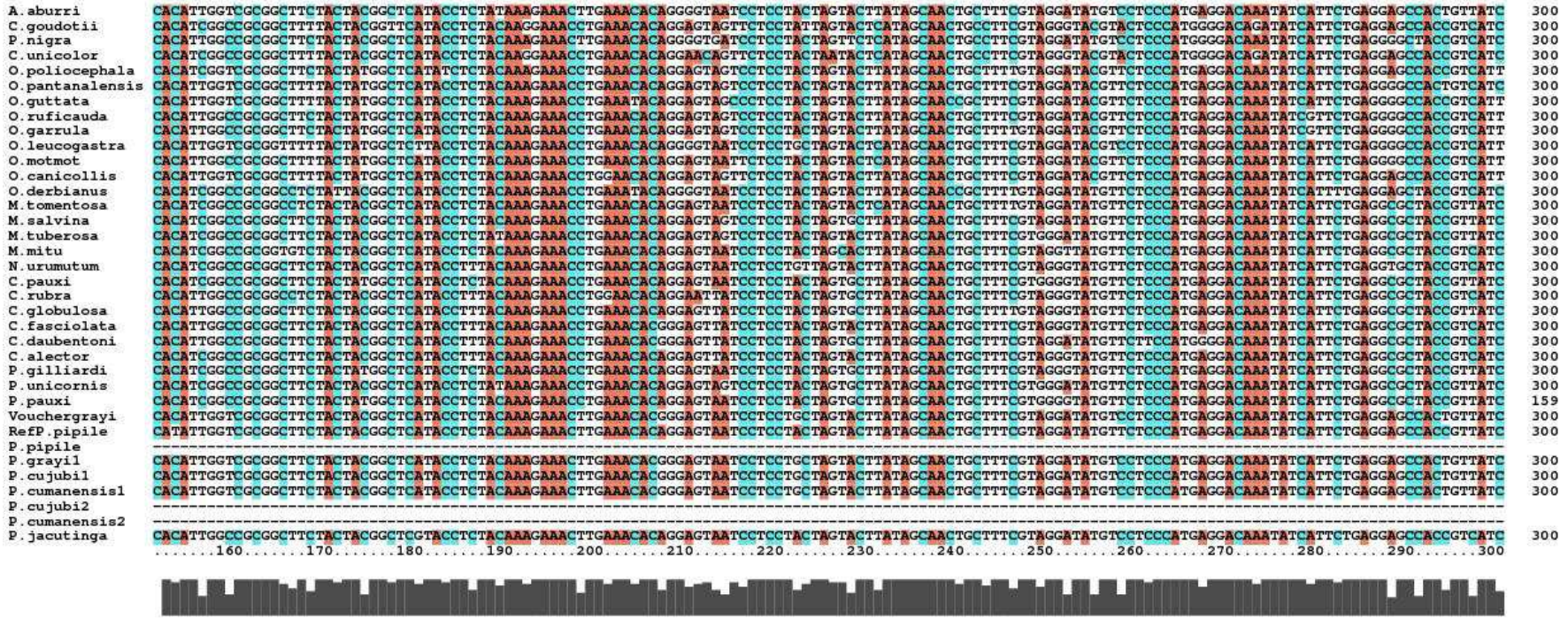


Appendices V

Cyt *b* alignment: Page 1 of 7

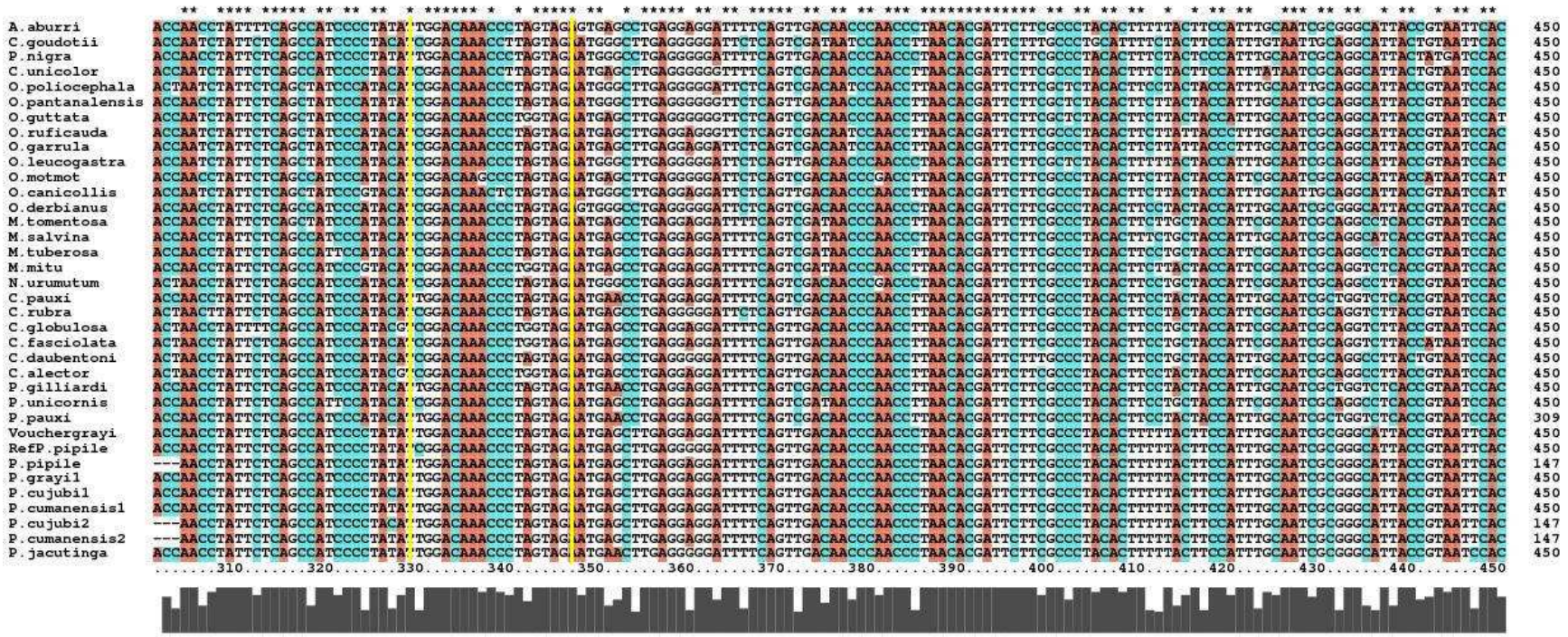


Cyt *b* alignment: Page 2 of 7



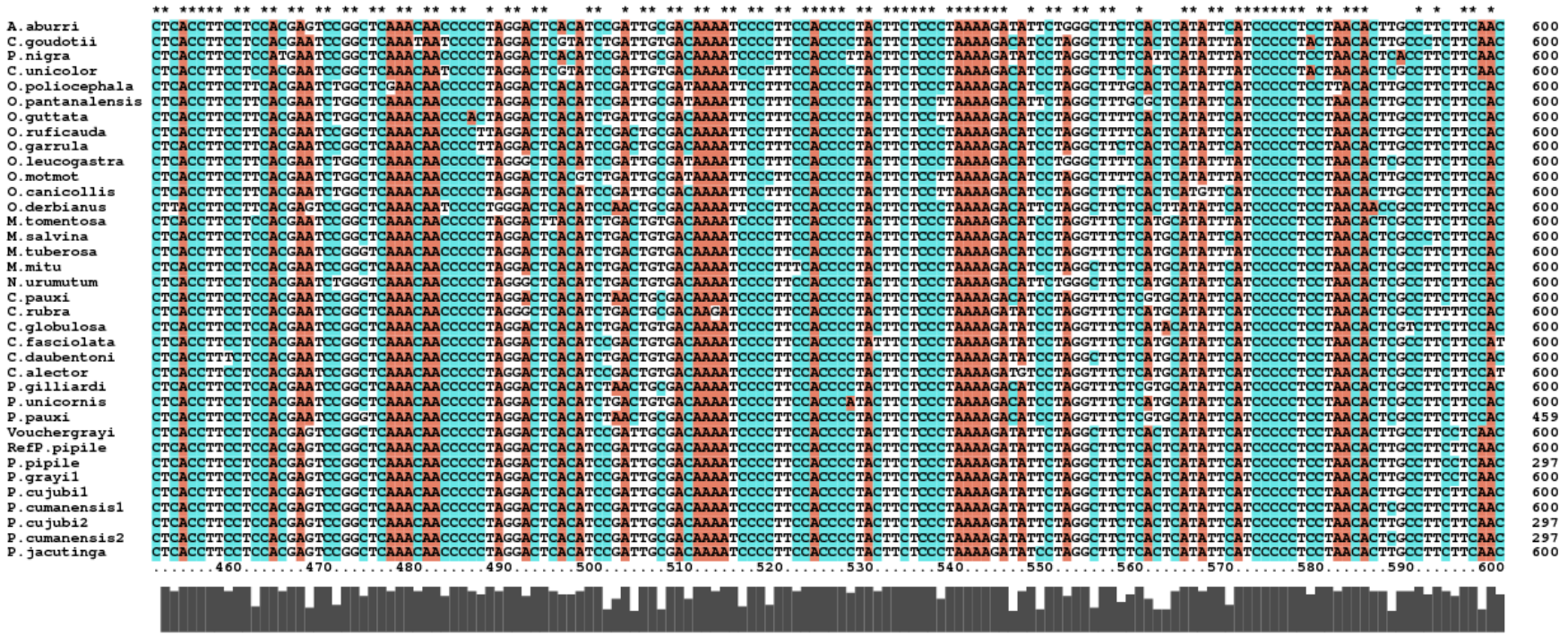
Appendices V

Cyt *b* alignment: Page 3 of 7



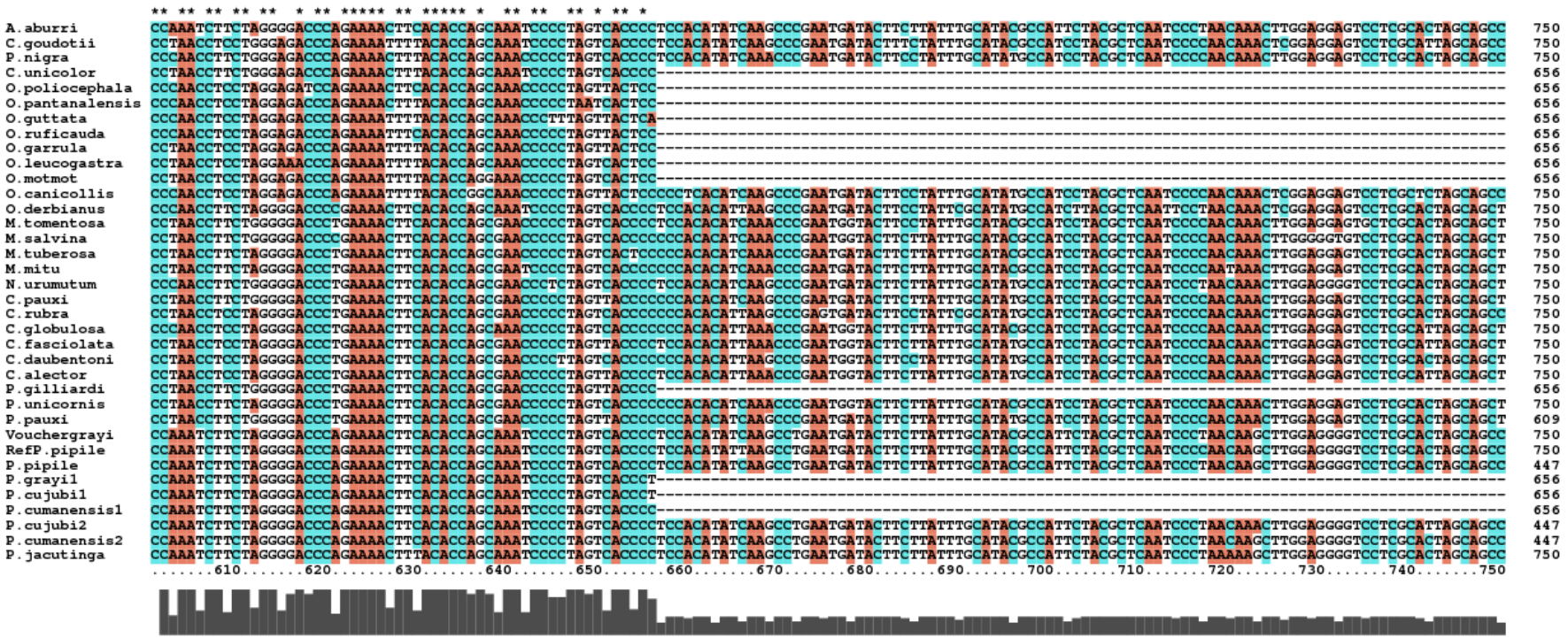
Appendices V

Cyt *b* alignment: Page 4 of 7



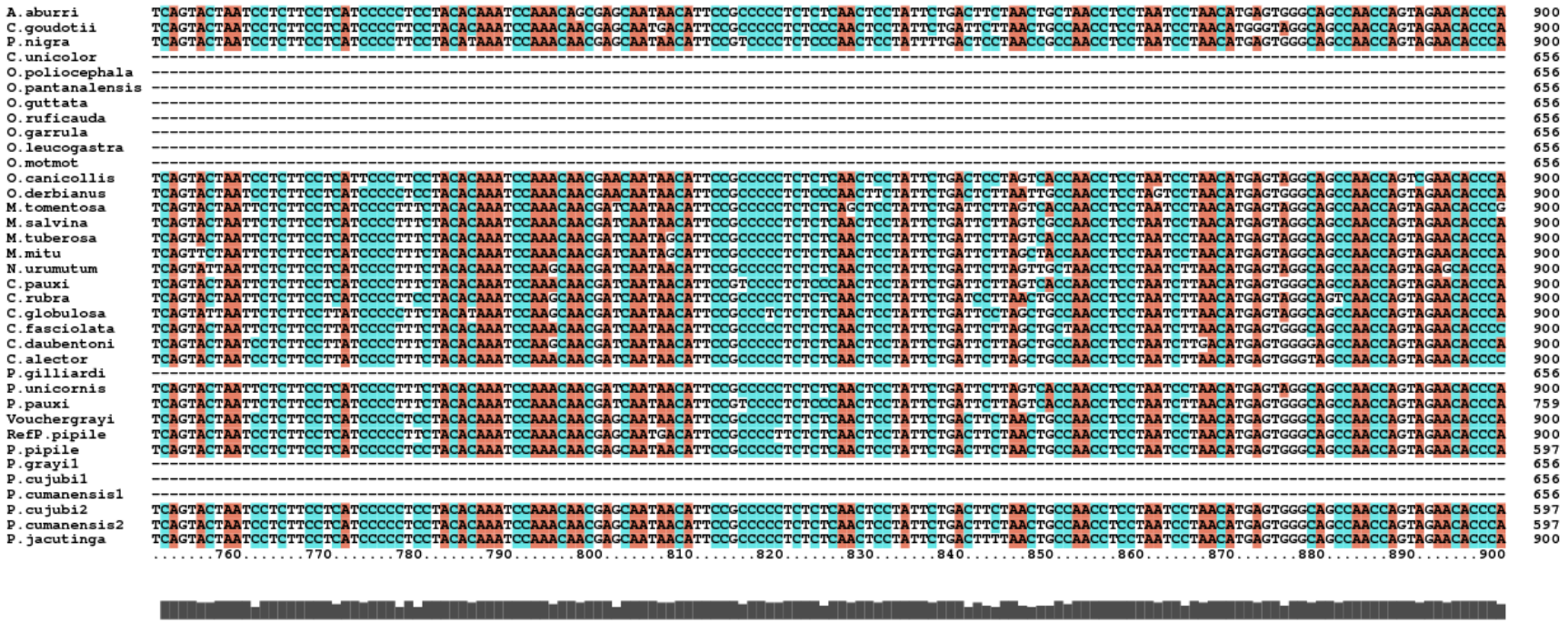
Appendices V

Cyt *b* alignment: Page 5 of 7



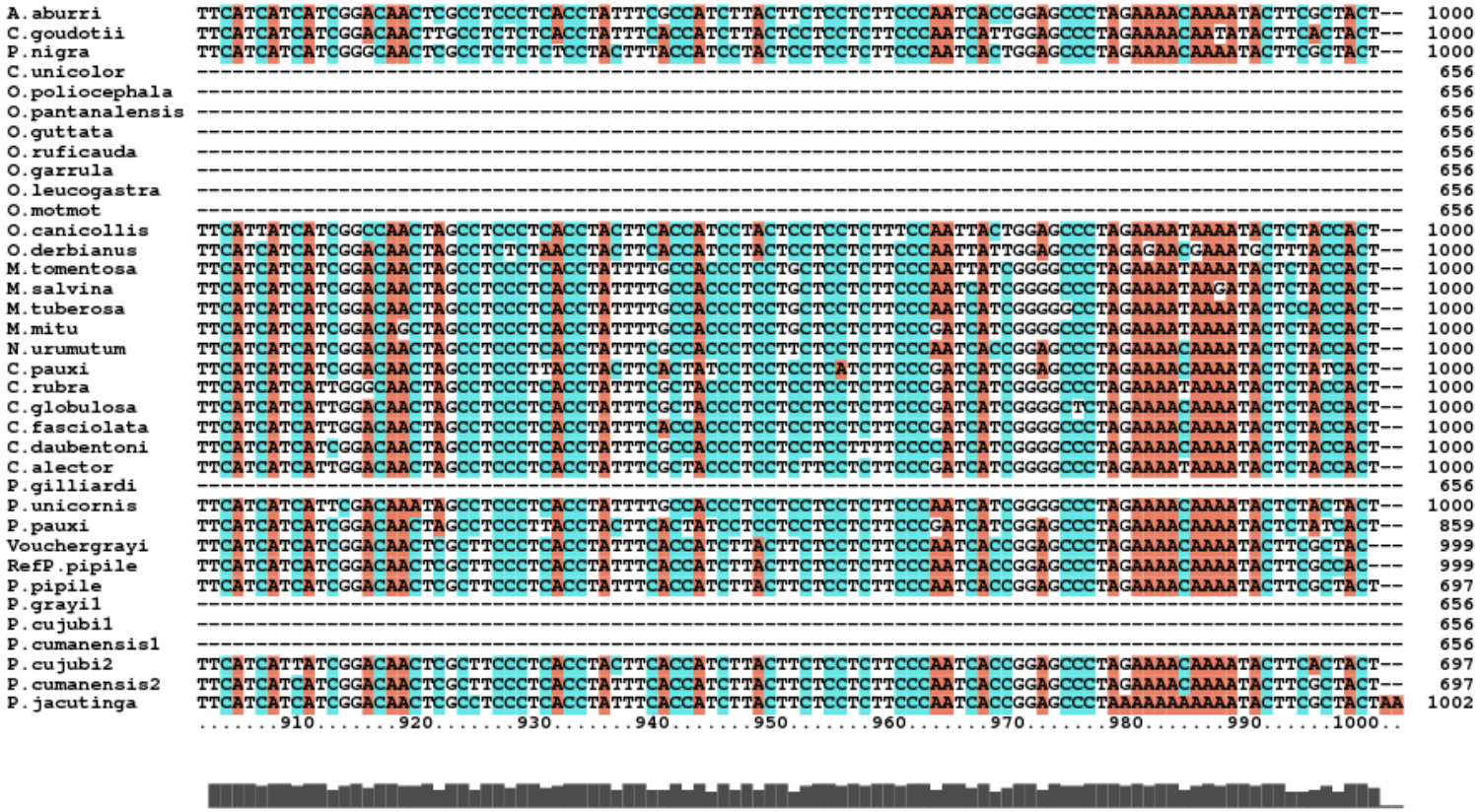
Appendices V

Cyt *b* alignment: Page 6 of 7



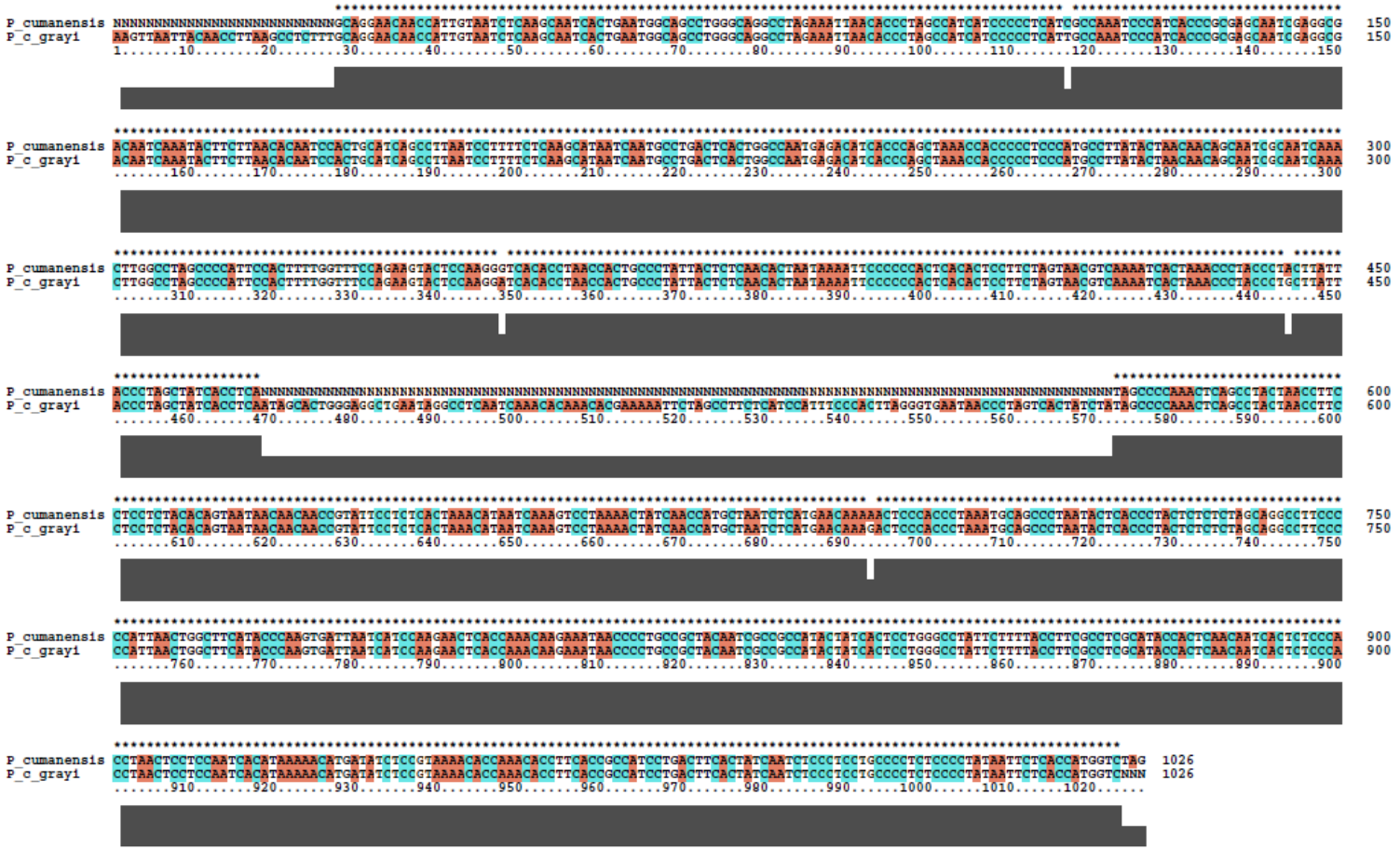
Appendices V

Cyt *b* alignment: Page 7 of 7



Appendices VI

ND2 Alignment of GenBank *Pipile cumanensis* data and voucher *Pipile cumanensis grayi* data obtained in the current study.



Appendices VII

Cyt *b* Alignment of GenBank *Pipile cumanensis* data and voucher *Pipile cumanensis grayi* data obtained in the current study.